

# BIOCONVERSIONS IN AQUEOUS TWO-PHASE SYSTEMS

*Elis Andersson*

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Department of Applied Microbiology



LUND 1986



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| <b>Title and subtitle</b><br>Bioconversions in aqueous two-phase systems.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                 |                            |
| <b>Abstract</b><br>Recirculation of biocatalysts in aqueous two-phase systems was investigated. Two model systems were studied: (i) recirculation of an enzyme, penicillin acylase (PA); and (ii) recirculation of living bacterial cells, <u>Bacillus</u> sp., for $\alpha$ -amylase production.<br>The recirculation of PA in aqueous two-phase systems was compared to other methods for recirculation, i.e. immobilization techniques and membrane reactors.<br>The stability of PA increased three times and the activity decreased 30% due to the conditions in the bottom phase of the aqueous two-phase system.<br>The production of $\alpha$ -amylase with cells of <u>Bacillus</u> sp., which were recirculated in aqueous two-phase systems, was compared to production with immobilized cells.<br>The amylase production of <u>Bacillus</u> sp. was influenced by the type of phase system used. Different strains of <u>Bacillus</u> sp. responded differently to the same phase system, giving increased or decreased amylase production. Solutions of poly(ethylene glycol) (PEG) 600 had properties similar to the phase systems. Depending on the strain of <u>Bacillus</u> sp. used, the amylase production increased or decreased.<br>Solutions of PEG changed the cell surface properties of <u>Bacillus</u> sp. |                                                                                                                                                                                 |                            |
| <b>Key words</b><br>bioconversions, aqueous two-phase systems, penicillin acylase, stability, activity, <u>Bacillus</u> sp., $\alpha$ -amylase, poly(ethylene glycol), cell properties                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                 |                            |
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*Elis Andersson*

Date April 10, 1986

Till mina föräldrar  
Till Håkan och Lennart

Everything should be as simple as it can be,  
but not simpler.

Albert Einstein



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# List of papers

This thesis is based on the following papers, referred to by their Roman numerals in the text.

- I. Enzymatic conversion in aqueous two-phase systems:  
deacylation of benzylpenicillin to 6-aminopenicillanic acid with penicillin acylase.  
Reproduced from Elis Andersson, Bo Mattiasson and Bärbel Hahn-Hägerdal, Enzyme and Microbial Technology, 1984, 6, 301-306, by permission of the publishers, Butterworth & Co (Publishers) Ltd. ©.
- II. Enzyme action in polymer and salt solutions:
  1. Stability of penicillin acylase in PEG and potassium phosphate solutions in relation to water activity.  
Elis Andersson and Bärbel Hahn-Hägerdal, submitted for publication.
- III. Enzyme action in polymer and salt solutions:
  2. Activity of penicillin acylase in PEG and potassium phosphate solutions in relation to water activity.  
Elis Andersson and Bärbel Hahn-Hägerdal, submitted for publication.
- IV.  $\alpha$ -Amylase production in aqueous two-phase systems with Bacillus subtilis.  
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- V.  $\alpha$ -Amylase production with Bacillus amyloliquefaciens in aqueous two-phase systems and PEG solutions.  
Elis Andersson and Bärbel Hahn-Hägerdal, submitted for publication.

# 1 Introduction

Biotechnological processes are based on the use of living microorganisms, enzymes from microorganisms or combinations of both. Living organisms are more favourable to use for the production of more complex products. The microbial products can be production of biomass, e.g. baker's yeast; extracellular products, e.g. enzymes and ethanol; or intracellular products, e.g. enzymes and vitamins. Enzymatic processes are favourable when the process only contains one single enzymatic conversion, e.g. isomerisation of glucose to fructose. Products, which are difficult or impossible to produce by traditional chemical means, can be produced using biotechnological processes. For example, the product can have a complex structure or have demands of being stereospecific. Biotechnical processes are, as other processes, dependent on their economical feasibility, which in the process is measured by the product concentration, overall productivity and conversion yield of the substrate.

Biocatalysts (microorganisms, enzymes) are often expensive. It is therefore desirable to be able to reuse the biocatalyst. It is also desirable to obtain a product stream, which is as pure and as concentrated as possible. During the last decades these demands have led to the development of several methods to reuse the biocatalyst. The most wellknown method is the immobilization technique, where the biocatalyst is bound to or encapsulated in a solid matrix composed of some polymer material (Methods of Enzymology, 1976). This has been successfully developed both for systems containing living cells, e.g. production of ethanol (Nagashima et al., 1984); and for systems based on enzymes, e.g. glucose isomerase and penicillin acylase (Godfrey, 1983). Recently, membrane reactors have been developed and commercialized for systems involving enzymes, e.g. amino acids (Leuchtenberger et al., 1984). Microbial cells can also be recirculated by

centrifugation in continuous processes, e.g. production of ethanol (Alfa-Laval, 1983). Another method for recirculation of biocatalysts is described and discussed in this thesis. It involves the use of aqueous two-phase systems, where the biocatalyst is present only in one of the two phases, thus making it possible to recirculate the biocatalyst and recover the product from the other, biocatalyst-free, phase.

Two different model systems are described in this thesis; one enzymatic process and one system where living microbial cells were used. Penicillin acylase (PA) was used for the deacylation of benzylpenicillin (BP) to 6-aminopenicillanic acid (6-APA) in the enzymatic system, and an extracellular starch degrading enzyme,  $\alpha$ -amylase, was produced with Bacillus sp. During the work, observations were made concerning the stability and activity of PA and the surface properties of Bacillus sp., which probably will be of importance for a technical exploitation of phase systems for recirculation of biocatalysts.



# 2 Penicillin acylase

Penicillin acylase (PA) was first described in 1950 by Sakagushi and Murao. The name of the enzyme has been discussed since its discovery (for a review, see Cole,1967). The name penicillin acylase will be used throughout this discussion. The formal name is penicillin amidohydrolase (EC 3.5.1.11).

## 2.1 PROPERTIES OF PENICILLIN ACYLASE

PA is commonly found in both bacteria and fungi. Among the bacteria it is particularly spread among Gram-negative bacteria, especially among members of Enterobacteriaceae. It is also common among actinomycetes. Many genera of fungi have been shown to contain penicillin acylases. These include Penicillium, Aspergillus, Trichophyton, Cephalosporium, Emericellopsis, Fusarium and Botrytis (Vandamme,1977).

In general, bacteria produce PA intracellularly. However, strains of Bacillus megaterium and Streptomyces lavendulae produce extracellular penicillin acylases. Most penicillin acylases produced by fungi are intracellular (Vandamme,1977). The molecular weights and  $K_m$ -values of some penicillin acylases from different organisms are shown in Table 2.1. PA from one organism is generally not a homogeneous enzyme. Kasche et al.(1984a) separated a PA preparation from E. coli into 5 different fractions with HPLC.

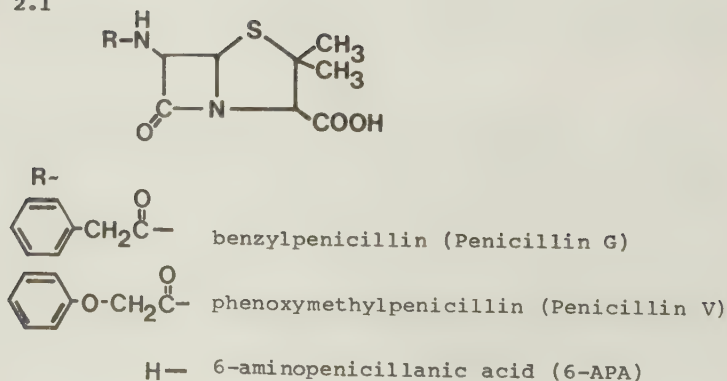
## 2.2 ACTIVITY OF PENICILLIN ACYLASE

The penicillin acylases can be divided into two groups according to their substrate specificity. Generally the fungal acylases hydrolyze phenoxymethylpenicillin (Penicillin V)

**Table 2.1** (for original references, see Vandamme, 1977)

|                             | Mw      | K <sub>m</sub> (mM) |
|-----------------------------|---------|---------------------|
| <u>Penicillin G acylase</u> |         |                     |
| <i>Escherichia coli</i>     | 70 000  | 0.02                |
| <i>Bacillus megaterium</i>  | 120 000 | 4.5                 |
| <i>Kluyvera citrophila</i>  | 63 000  | 1.4                 |
| <u>Penicillin V acylase</u> |         |                     |
| <i>Erwinia aroidae</i>      | 62 000  | 35                  |
| <i>Fusarium semitectum</i>  | 67 000  | 4.75                |

**Figure 2.1**



faster than BP(benzylpenicillin, Penicillin G) (Figure 2.1). The majority of the bacterial acylases hydrolyze BP faster than phenoxymethylpenicillin. The hydrolysis product in both cases is 6-aminopenicillanic acid (6-APA). Penicillin acylases are not specific for penicillins, but have instead specificity for certain types of acyl groups occurring in penicillins. The reaction is generally an equilibrium and PA can also be used for the synthesis of penicillins.



The hydrolytic activity of PA is optimal at alkaline pH values between 7.5 and 9.0, while the synthetic activity is optimal at acid pH between 5.0 and 6.0.

It has been shown for an Escherichia coli acylase, that it had hydrolytic activity towards 14 different penicillins among which p-hydroxybenzylpenicillin (Penicillin X) was the best substrate (Cole,1969a). For the same PA, Cole (1969b) also investigated substrate specificity for the synthetic reaction to produce BP from 6-APA. 12 different phenylacetic acid derivatives could all be used as a substrate, and N-phenylacetyl glycine was the best substrate. The synthesis of penicillin can, due to the low substrate specificity, be performed both as an equilibrium and a kinetically controlled synthesis (Kasche et al.,1984b).

The hydrolytic activity of PA from E.coli is inhibited by high concentrations of the substrate BP. Both the products also inhibit the enzyme, 6-APA noncompetitively and phenylacetic acid competitively (Balasingham et al.,1972).

The kinetics of PA have been studied mainly for the synthetic reaction. Two mechanisms have been proposed (Kasche et al., 1984a; for a more extensive review, see Kasche,1986).

It was earlier suggested that the enzyme follows a mechanism that has been established for serine proteases ( $\alpha$ -chymotrypsin, trypsin) and S-H protease (papain) (Balasingham et al.,1972; Kutzbach and Rauenbusch,1974; Svedas et al.,1980; Konecny et al.,1981). This mechanism (Figure 2.2A) involves

a covalent acyl-enzyme (E-PhAc) as an intermediate, that can be deacylated by either  $H_2O$  or any other nucleophile (e.g. 6-APA). If this mechanism applies, the deacylation rate constant  $k'_3/k_3$  should be independent of the leaving group (glycine) and the nucleophile concentration (6-APA). However, such a dependence has been observed for PA (Klesov et al., 1977; Cole, 1969c). Therefore another mechanism was later proposed for PA (Kasche et al., 1984a), which was established for proteases and  $\beta$ -galactosidase (Figure 2.2B). Here, the nucleophile (6-APA) is bound to the acyl-enzyme prior to the deacylation. The products formed are also substrates for PA, which means that the same intermediates are involved in both the formation and the hydrolysis of these compounds. This is described in the nucleophile binding mechanism (Figure 2.2B) but not in the direct deacylation mechanism (Figure 2.2A). Experimental evidence for the latter theory has been presented (Kasche et al., 1984a).

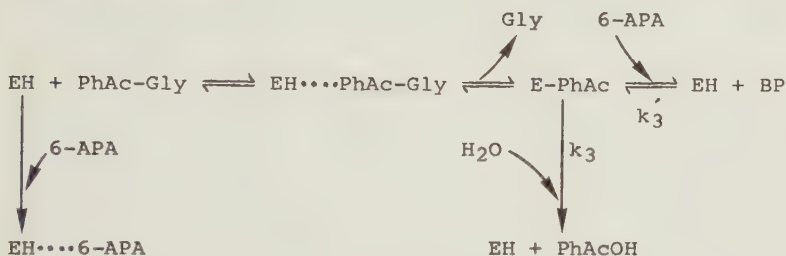
### 2.3 CHOICE OF MODEL SYSTEM

The hydrolytic reaction of PA is product inhibited (Balasingham et al., 1972) and an important process with immobilized PA. Therefore this reaction was chosen as a model system for studies in aqueous two-phase systems.

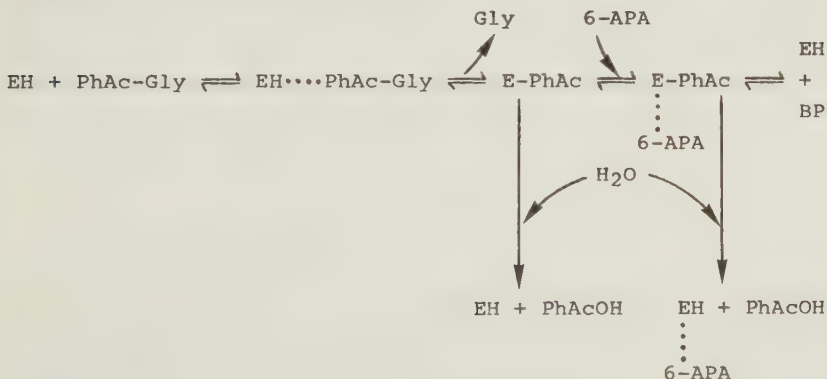


**Figure 2.2** Mechanism of the hydrolase catalysed group transfer reaction : N-Phenylacetyl-glycine + 6-APA  $\rightleftharpoons$  BP + Glycine with an acyl enzyme (PA) that is deacylated by the nucleophiles  $H_2O$  or 6-APA. This figure is adopted from the general mechanism (Kasche *et al.*,1984a) and described for the kinetically controlled synthesis. Symbols: EH = enzyme(PA), PhAc-Gly = activated acyl doner, Gly = leaving group, E-PhAc = acyl-enzyme, 6-APA = nucleophile, PhAcOH = hydrolysis product and BP = semisynthetic product.

**A)** Mechanism with only one acyl-enzyme for which the nucleophiles compete.



**B)** Mechanism where the nucleophile 6-APA is bound to the acyl-enzyme before it can deacylate it to form BP. Both acyl-enzymes can be deacylated by  $H_2O$ .





# 3 Bacillus

## 3.1 GENUS BACILLUS

The genus Bacillus belongs to the family Bacillaceae, which contains the genera with Gram-positive endospore forming species, e.g. Bacillus, Clostridium, Desulfotomaculum and Sporosarcina (Bergey's Manual of Determinative Bacteriology, 1974),

Bacillus means a small rod, and the cells belonging to this genus are rod-shaped. Most of them are also motile, having a lateral flagella. They are strict aerobes or facultative anaerobes and catalase is usually produced. The endospores formed are heat resistant and only one spore is formed in each cell. However, nonspore forming mutants are often used for production of extracellular enzymes.

Some properties for three starch degrading bacilli are shown in Table 3.1. The information is limited for B. amyloliquefaciens. Many strains belonging to this species, have previously been named B. subtilis (Bergey's Manual of Determinative Bacteriology, 1974) and have not been carefully taxonomically investigated.

## 3.2 EXTRACELLULAR ENZYMES

According to Priest (1984), extracellular enzymes are in a broad sense enzymes that cross the cytoplasmic membrane and exist in all kinds of organisms. In bacteria, strains of Bacillus, Clostridium, Cytophaga and many actinomycetes produce extracellular enzymes. In a strict sense, according to the definition above, many enzymes in Gram-negative bacteria are also extracellular as they have crossed the cytoplasmic

**Table 3.1** Amylase producing Bacillus sp.

|                                           | <u>Bacillus</u><br><u>subtilis</u> | <u>Bacillus</u><br><u>licheniformis</u> | <u>Bacillus</u><br><u>amylolique-</u><br><u>faciens</u> |
|-------------------------------------------|------------------------------------|-----------------------------------------|---------------------------------------------------------|
| Rods, width(μm)                           | 0.7-0.8                            | 0.6-0.8                                 |                                                         |
| length(μm)                                | 2-3                                | 1.5-3                                   |                                                         |
| Motile                                    | +                                  | +                                       |                                                         |
| Starch as substrate                       | +                                  | +                                       | +                                                       |
| Anaerobic agar                            | -                                  | +                                       | -                                                       |
| $\text{NO}_3^- \rightarrow \text{NO}_2^-$ | +                                  | +                                       |                                                         |
| Spore position                            | Central                            | Central                                 | Central                                                 |
| T <sub>max</sub> (°C)                     | 45-55                              | 50-55                                   |                                                         |
| T <sub>min</sub> (°C)                     | 5-20                               | 15                                      |                                                         |
| G+C (%)                                   | 42-43                              | 43-47                                   | 43-45                                                   |

membrane into the periplasmic space between the two cell membranes.

The most important commercial enzymes are Bacillus proteases, bacterial amylases, glucoamylases (fungi) and glucose isomerases (bacteria). These enzymes constituted 96% of the enzyme world market in 1980. All of these microbial enzymes except glucose isomerase are extracellular.

The theory for the transport of extracellular enzymes is based on the signal hypothesis, which has been illustratively reviewed and refined by Randall and Hardy (1984). The transport across the membrane can occur both as co-translational and post-translational secretion.

The precursor forms of secretory proteins usually contain an NH<sub>2</sub>-terminal extension of some 15 to 32 amino acids, the signal peptide. As the signal peptide emerges from the ribosome, it interacts with the inner surface of the cytoplasmic membrane in bacteria or with the endoplasmic reticulum in eukaryotes. As the polypeptide is synthesized in the ribo-

some, it passes through the membrane. When the synthesis is finished, a signal peptidase removes the signal peptide on the outside of the membrane, thus allowing the protein to obtain its active conformation on the outside of the membrane. This is called co-translational secretion, and has been established for  $\alpha$ -amylase in B. subtilis,  $\beta$ -lactamase in B. licheniformis and the toxin of Corynebacterium diphtheriae (Davis and Tai,1980).

It has been demonstrated that several proteins are incorporated into and transported across membranes after they have been synthesized. There are several examples of this post-translational secretion among bacteria. Part of the cholera protein is synthesized intracellularly, as well as  $\alpha$ -glucosidase in some Bacillus strains. It is supposed that proteins which are post-translationally secreted, also have a signal sequence which facilitates the membrane transport.

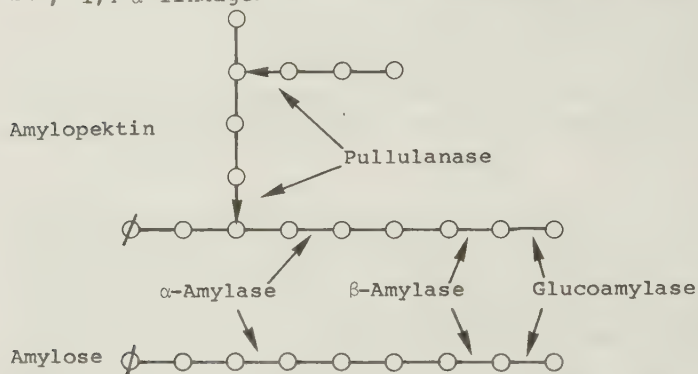
Enzymes produced at constant rate, irrespective of the presence of substrate in the environment, are called constitutive. Several extracellular enzymes are constitutive, like amylases and proteases of Bacillus amyloliquefaciens and Bacillus licheniformis (Priest,1984). However, enzyme synthesis is usually inducible. Inducible enzymes are produced at a low basal rate, when no substrate is available. When substrate is present in the medium, the synthetic rate of the enzyme increases dramatically. For extracellular enzymes, the induction is more complicated, as the cell has to monitor the substrate level in the surrounding medium. It has been proposed, that the inducible enzyme is secreted in a low constitutive level. In the presence of substrate, low molecular weight products accumulate. These can enter the cell, thus effecting the induction (Priest,1984).

### 3.2.1 Starch degrading enzymes

The starch hydrolyzing enzymes is a major group among the industrially important enzymes. The different enzymes involved in starch hydrolysis and their action on the amylopectin and amylose molecules are shown in Figure 3.1.



**Figure 3.1** Starch hydrolyzing enzymes. (○), glucose residue; (Ø), glucose with a reducing group. → 1,6- $\alpha$ -linkage; —, 1,4- $\alpha$ -linkage.



$\alpha$ -Amylase is commercially produced mainly with Bacillus strains (for a review over amylases, see Fogerty, 1983). The  $\alpha$ -amylase of Bacillus amyloliquefaciens has a molecular weight of 50,000 dalton and a pH optimum of 5.9. It is a calcium enzyme, where the calcium is needed both for activity and for increased stability. It can be used in starch hydrolysis at temperatures approaching 90°C.

The  $\alpha$ -amylase from B. licheniformis can be used for starch hydrolysis at the gelatinization temperature (105-110°C). It is larger than the B. amyloliquefaciens enzyme (60.000 dalton) and has a lower requirement for  $\text{Ca}^{2+}$ .

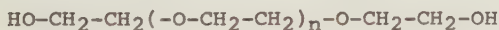
### 3.3 CHOICE OF MODEL SYSTEM

The production of  $\alpha$ -amylase with Bacillus sp. was chosen as model system for production in aqueous two-phase systems.  $\alpha$ -amylase is an extracellular enzyme of great industrial importance and due to its macromolecular nature, it ought to be possible to successfully partition the enzyme to a cell-free product phase.

# 4 Aqueous two-phase systems

Phase separation is a general phenomenon, which occurs when two solutions of water-soluble polymers are mixed. The phenomenon has been known for a long time (Beijerinck, 1896). Alternatively to the use of two polymers, one polymer and one salt solution can be used to form an aqueous two-phase system. The two most commonly explored polymers are poly(ethylene glycol) (PEG) and dextran. However, many other polymers have been shown to form phase systems (Albertsson, 1971). Among the polymer/salt systems, PEG/potassium phosphate and PEG/magnesium sulfate are most frequently described.

Both natural polymers and synthetic polymers can be used to obtain phase systems. The frequently used PEG is a linear synthetic polymer, molecular weights from 200 to 40 000 can be obtained. PEG has the following general formula:



Dextran is a natural polymer produced by a lactic acid bacteria, Leuconostoc mesenteroides. It is a polyglucose with mainly  $\alpha$ -1 $\rightarrow$ 6 linkages and it is branched through  $\alpha$ -1 $\rightarrow$ 3 linkages. PEG has a more hydrophobic character than dextran (Albertsson, 1971).

The principle for separation of molecules in aqueous two-phase systems is the nonuniform distribution of substances between the two phases. This is described by the partition coefficient,  $K$ , which is defined as the ratio of the concentrations in the top and the bottom phase, respectively.

$$K = C_T / C_B$$

The observed partition coefficient is an integral of several interacting properties and can be expressed:

$$\ln K = \ln K_{el} + \ln K_{hydrophob} + \ln K_{hydrophil} + \ln K_{conf} + \dots$$

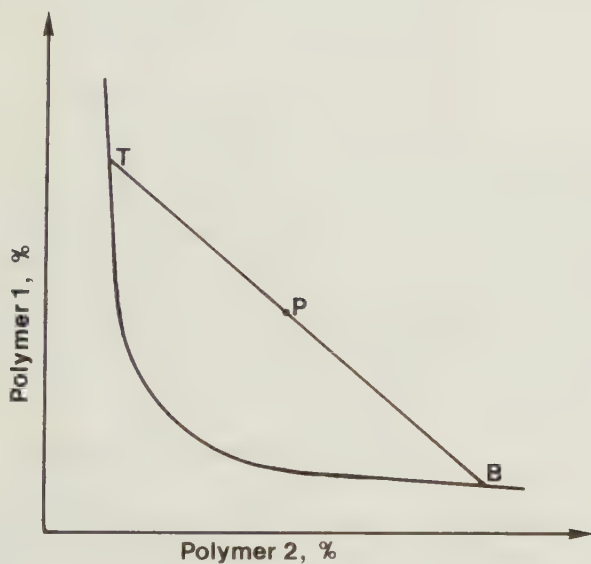
where  $K_{el}$ ,  $K_{hydrophob}$ ,  $K_{hydrophil}$  and  $K_{conf}$  describe partition factors due to electric, hydrophobic and hydrophilic forces or conformational effects, respectively (Albertsson, 1977). As the surface properties of small molecules, macromolecules, cell organelles and cells determine the distribution in aqueous two-phase systems, the partition can most easily be altered by manipulating the three first parameters, i.e. by changing the charge or by including more hydrophobic and hydrophilic polymers into the phase systems.

Under given conditions, an aqueous two-phase system can be described by a phase diagram. The system is actually a tertiary system, but for simplicity phase diagrams are drawn as two-component systems as in Figure 4.1. The tie-line ( $\overline{TB}$ ) relates to the mass ratio between the two phases. Thus, the expression can be written as  $\text{Mass}_T/\text{Mass}_B = \overline{PB}/\overline{PT}$  where  $\overline{PT}$  and  $\overline{PB}$  are the distance between the indicated points on the tie-line. Practically, the mass ratio is considered equal to the volume ratio ( $V_T/V_B$ ).

Aqueous two-phase systems have several properties which make them suitable to use together with biological material. The water content of the two phases exceeds 75%, although the two phases are no longer miscible.

The surface tension between the phases is very low, 0.0001 - 0.1 mN/m (Albertsson, 1971), as compared to interfacial tensions between water and an organic solvent, which usually are in the range 5-50 mN/m (Shaw, 1970). However, the low interfacial tension increases the separation time for the phases, which is a disadvantage in extraction and purification of a product. It can, however, be expected to be an advantage for the mass transfer between the phases, since a very large interfacial area is formed.

The introduction of polymers into the solution increases the viscosity. The viscosity differs quite markedly between the two phases. For a phase system composed of PEG 3350, 9% w/w and Dextran T500, 2% (w/w), the viscosity of the bottom phase was 93,9 mPa·s and in the top phase it was 3,0 mPa·s (Kroner et al., 1982). On the other hand, for a phase systems composed of PEG 20M, 8,9% (w/w) and potassium phosphate, 7,6% (w/w) the top phase had a viscosity of 136 mPa·s and the bottom phase had a viscosity of 2.5 mPa·s (Anneli Svensson, personal communication).



**Figure 4.1** Phase diagram for two polymers, 1 and 2, and water. Polymer concentration represented by points above the binodal curve gives two-phase systems. Below the curve the solutions are homogeneous.





# 5 Aqueous two-phase systems in biotechnology

This presentation of the use of aqueous two-phase systems in biotechnology will be limited to: analytical applications, partition of proteins and cells and bioconversions. The use of aqueous two-phase systems as a scientific tool will thus be left outside this presentation.

## 5.1 ANALYTICAL APPLICATIONS

An analytical technique has been developed, where aqueous two-phase systems are used to separate different components in the assay. This method has been named Partition Affinity Ligand Assay (PALA) (Mattiasson and Ling,1980). The assay can be performed in two different ways, either as a direct binding assay or as a competitive binding assay for the determination of cell numbers (Mattiasson et al.,1981).

## 5.2 PARTITION OF PROTEINS AND CELLS

### 5.2.1 Extraction and purification of proteins

Extraction and purification of intracellular microbial proteins based on the partition of proteins in aqueous two-phase systems has during recent years been developed to a powerful method (for a review, see Kula et al.,1982). Compared to other purification techniques, the scale-up is easy and precise, and high capacity, high product yields and high potential for continuous operation can be obtained(Kroner et al.,1984).

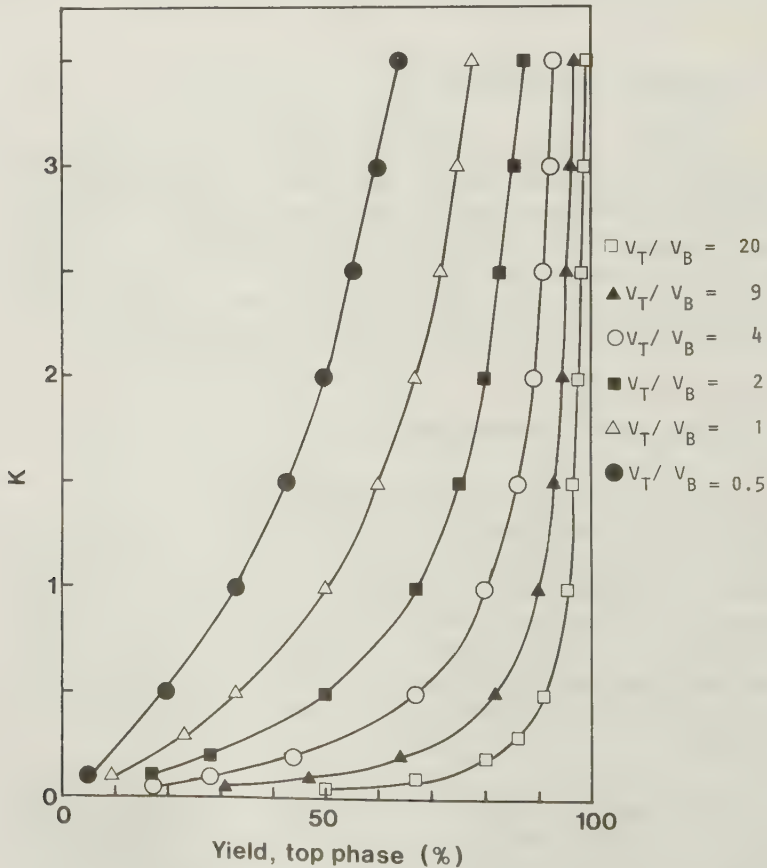
In protein purification the separation of the cell debris from the protein is what is desired. This can be accomplished by partition of the protein and cell debris to opposite phases. Due to their size, cells and cell debris are often

observed to have a onesided partition. Frequently, the cell debris is removed with the bottom phase of the aqueous two-phase system (Kula et al.,1982). Thus, for an efficient separation, the protein should be partitioned to the top phase. The yield of the protein in the top phase can be calculated by the expression:

$$Y_T = 100 \cdot V_T \cdot K / V_T \cdot K + V_B$$

where  $V_T$  and  $V_B$  are the volumes of the top and bottom phase, respectively. Thus, the yield is influenced both by the

Figure 5.1



partition coefficient,  $K$ , and the volume ratio ( $V_T/V_B$ ). The importance of the volume ratio is shown grafically in Figure 5.1. Thus, with a volume ratio of 1.0,  $Y_T$  increases from 50% to 66.7% when  $K$  increases from 1.0 to 2.0. On the other hand, if  $K = 1.0$  and the volume ratio increases from 1.0 to 9.0, the yield increases from 50% to 90%. Thus, both a favourable  $K$  and a favourable volume ratio are important for the yield in the protein extraction.

### 5.2.2 Partition of penicillin acylase

The concept in the present investigation was to recirculate the enzyme penicillin acylase (PA) in a bottom phase of an aqueous two-phase system. Contrary to obtaining a high product yield in the top phase (Figure 5.1), the recirculation of an enzyme in a bottom phase demands a low  $K$  and a low volume ratio. It was calculated that with  $K = 0.04$  and a volume ratio of 1.0, 3.8 % of the enzyme is lost to the top phase. If the volume ratio is increased to 2.0 the corresponding loss is 7.4 %. This will eventually lead to a wash-out of the enzyme.

First, the partition of PA to the bottom phase of aqueous two-phase systems, composed of PEG and dextran, was investigated (Table 5.1)(Paper I). The partition coefficients did not fulfill the demands of being sufficiently onesided.

The partition was therefore also investigated in phase systems composed of PEG and salts (Table 5.2)(Paper I). PA had an unusually low  $K$ , i.e. a very onesided partition to the bottom phase, in all the phase systems investigated. Data for partition in PEG/salt systems for other enzymes, which were optimized for purification, show a preference in partition to the top phase with partition coefficients between 1 and 20 (Veide et al., 1983, Kroner et al., 1984). This points out the importance of the physico-chemical properties of the proteins for the partition in aqueous two-phase systems.

The PEG/potassium phosphate system with  $K < 0.01$  was chosen for the conversion experiments, while this system has a good buffering capacity and does not rapidly denature the enzyme,

**Table 5.1** Partition of PA in aqueous two-phase systems composed of PEG and dextran. All data at pH 7.8 and 22°C. K is based on activity measurement and is defined as the activity in the top phase divided by the activity in the bottom phase (from Paper I).

| PEG type | % (w/w) | Dextran type | % (w/w) | Buffer              | Molarity | K    |
|----------|---------|--------------|---------|---------------------|----------|------|
| 8000     | 7.5     | T40          | 6.0     | Tris                | 0.5      | 0.14 |
| 8000     | 7.5     | T40          | 6.0     | Tris                | 0.3      | 0.13 |
| 8000     | 7.5     | T40          | 6.0     | Sodium phosphate    | 0.2      | 0.10 |
| 8000     | 7.5     | T40          | 6.0     | Sodium phosphate    | 0.5      | 0.04 |
| 8000     | 7.5     | T40          | 6.0     | Potassium phosphate | 0.5      | 0.35 |
| 8000     | 7.5     | T40          | 6.0     | Sodium phosphate    | 0.05     | 0.15 |
| 20000    | 10.0    | T10          | 5.0     | Sodium phosphate    | 0.12     | 0.03 |

**Table 5.2** Partition of PA in aqueous two-phase systems composed of PEG and salt. The experimental conditions are the same as in Table 5.1 except for phase system asterisked, where the temperature was 37°C. This phase system is formed at a temperature of 25°C (from Paper I).

| PEG type | % (w/w) | Salt type           | % (w/w) | Phase volume ratio (top/bottom) | K         |
|----------|---------|---------------------|---------|---------------------------------|-----------|
| 8000     | 10.0    | Potassium phosphate | 10.0    | 0.69                            | 0.02-0.03 |
| 20000    | 10.0    | Potassium phosphate | 10.0    | 0.64                            | 0.02-0.03 |
| *20000   | 8.9     | Potassium phosphate | 7.6     | 0.88                            | <0.01     |
| 3350     | 12.0    | Magnesium sulfate   | 10.0    | 0.43                            | <0.01     |

which occurred in the PEG/magnesium sulfate system (Paper I). This phase system, however, has the disadvantage of being temperature unstable and is only formed at temperatures above 25°C.

### 5.2.3 Partition of $\alpha$ -amylase

When  $\alpha$ -amylase is produced by Bacillus sp. in aqueous two-phase systems, the cells are expected to partition to the bottom phase. Therefore, phase systems were looked for, which gave a high yield of the enzyme in the top phase. When  $\alpha$ -amylase should be recovered from the top phase, the concept of enzyme recovery in the top phase could be used (Figure 5.1).

The partition coefficient, K, has been reported to be 3.64 for  $\alpha$ -amylase in a phase system of PEG and dextran (Kroner et al., 1982). This phase system contained 0.3 M potassium phosphate, pH 7.5. Phosphate anions form complexes with dextran in alkaline solutions. This gel formation increases K for many proteins. For the purification of enzymes, K can be increased in this way. However, high pH and a high ionic strength are not a proper environment for enzyme producing microbial cells. In the presence of phosphate, intensive stirring and aeration further increases the gel formation (Paper IV). This makes it impossible to use high phosphate concentrations during a fermentation in order to increase the yield of the  $\alpha$ -amylase in the top phase. Therefore, the phosphate concentration was decreased from 0.3 to 0 M, which also decreased K markedly from 3.64 to 0.33 (Figure 5.2). However, due to the favourable volume ratio, 8.8, the diminished K only decreased the yield of the enzyme in the top phase from 96.7% to 72.9%.

By partly replacing the PEG 3350 in the phase system with PEG 600, K can be increased to 0.5 (Figure 5.3). This increases the enzyme yield to 82% in the top phase. This phase system, with a volume ratio of 9.0 and contents of PEG 600, 8% (w/w), PEG 3350, 5% (w/w) and Dextran T500, 2% (w/w), was used for the production experiments.

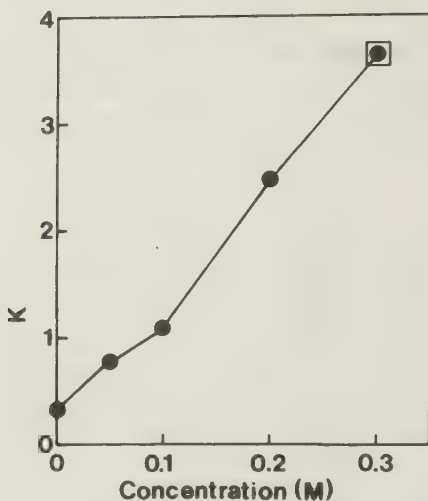


Figure 5.2 Partition coefficient,  $K$ , for  $\alpha$ -amylase in a phase system of PEG 3350, 9%(w/w), Dextran T500, 2%(w/w), pH 7.5, with various potassium phosphate concentrations. ( $\square$ ) Value from Kroner *et al.*, 1982. (from Paper IV).

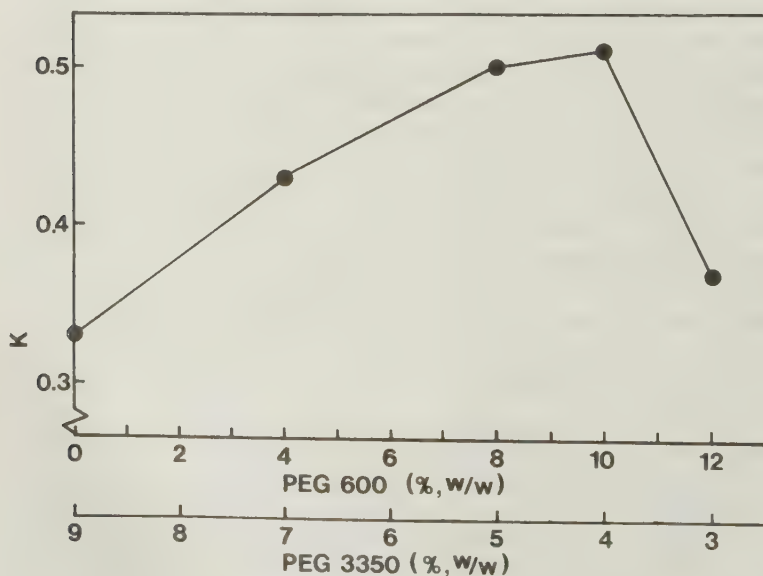


Figure 5.3 Partition coefficient,  $K$ , for  $\alpha$ -amylase in a phase system of Dextran T500, 2%(w/w) and different concentrations of PEG 600 and PEG 3350, pH 7.0 (from Paper IV).



#### 5.2.4 Partition of cells

The partition of cells in aqueous two-phase systems is determined by the surface properties of the cells. The interfacial energy of the cells can be determined by measuring the contact angles between the cells and the medium in an aqueous two-phase system (Schürch et al.,1981). Another method of measuring the cell surface energy is to measure the partition coefficient of the cells in an aqueous two phase system. This has been described for mammalian (Gerson,1980) and bacterial cells (Gerson and Akit,1980).

The latter method was used to partition cells of Bacillus amyloliquefaciens and Bacillus subtilis in different aqueous two-phase systems (Table 5.3)(Paper V). Cells grown in a normal growth medium were compared to cells grown in a 20% (w/w) PEG 600 medium. The latter medium was chosen, because it exclusively increased the  $\alpha$ -amylase production with B. subtilis (Ramgren et al.) and repressed the production with B. amyloliquefaciens (Paper V). The reference cells partitioned mainly to the bottom phase and the interphase. When grown in the PEG medium, the cells of B. amyloliquefaciens partitioned to a higher extent than the reference cells to the top phase, indicating that part of the cell population had more hydrophobic cell surfaces. Contrary, the cells of B. subtilis grown in the PEG medium partitioned to a higher extent than the reference cells to the bottom phase, indicating a cell population with more hydrophilic cell surfaces.

Also in the production systems, the cells of Bacillus subtilis and Bacillus amyloliquefaciens behaved differently. The cells of B. subtilis partitioned to the bottom phase (Paper IV)(cover photography of this thesis). In the same phase systems, cells of B. amyloliquefaciens partitioned only partially to the bottom phase and as the fermentation proceeded, the cells increasingly partitioned to the top phase (Paper V). In another study with seven different strains of B. subtilis (Kantelinén et al.), the different strains showed a varied partition pattern with partition either to the bottom phase, the bottom phase + the interphase or the interphase + the top phase. One strain, which partitioned totally to the bottom phase, even moved almost completely to the top

phase when the fermentation proceeded. This leakage to the product phase is a disadvantage, which has to be considered, when a cell-free product phase is desired.

This indicates that both aqueous two-phase systems and PEG can change the cell surface, probably by a direct interaction between PEG and the cell membranes and/or the cell walls.

**Table 5.3**

Partition of Bacillus amyloliquefaciens and Bacillus subtilis in aqueous two-phase systems at 25°C (from Paper V).

Polymer concentration:

(PEG 3350 / Dextran T500)(%,w/w) 6/6      7/7      8/8

Bacillus amyloliquefaciens

Reference sample

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 8.1  | 8.3  | 7.7  |
| Bottom phase (%) | 35.5 | 47.5 | 55.8 |

PEG grown cells

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 61.1 | 34.3 | 17.0 |
| Bottom phase (%) | 8.1  | 10.9 | 45.1 |

Bacillus subtilis

Reference sample

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 2.9  | 2.8  | 1.0  |
| Bottom phase (%) | 20.2 | 25.5 | 35.3 |

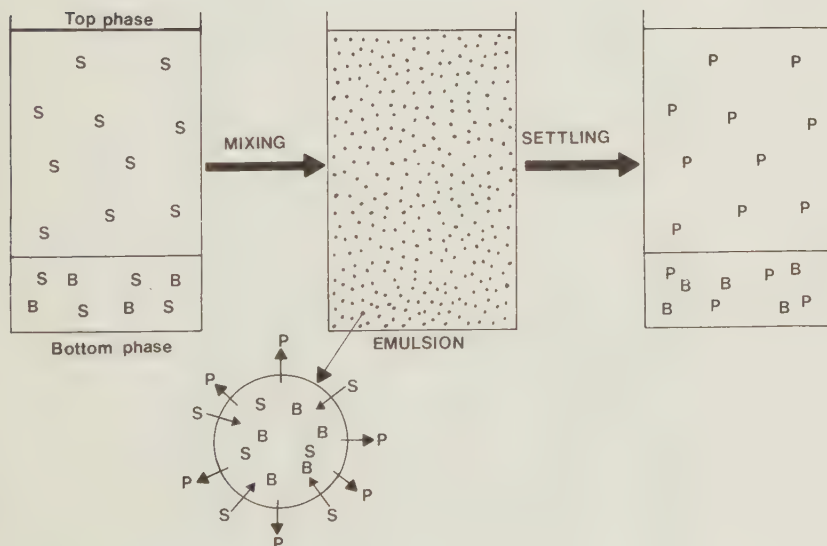
PEG grown cells

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 2.1  | 2.1  | 1.3  |
| Bottom phase (%) | 41.3 | 52.9 | 54.6 |

### 5.3 BIOCONVERSIONS

Bioconversions in aqueous two-phase systems have been described for systems involving enzymes, cell organelles, living cells and simultaneous use of enzymes and cells. A model for partition of the biocatalyst to the bottom phase and the product to the top phase has been adopted in all conversion studies, except one (Kuhlmann et al.,1980). This model is illustrated in Figure 5.4.

Enzymatic conversions in aqueous two-phase systems are summarized in Table 5.4. The difficulty to partition the enzyme totally to one phase, together with the difficulty to partition the product to the opposite phase is evident. One way to overcome this is to combine a phase system with an ultra-filtration membrane unit (Tjerneld et al.,1985c).



**Figure 5.4** Bioconversions in aqueous two-phase systems. B; biocatalyst, S; substrate, P; product.

Table 5.4 Enzymatic bioconversions in aqueous two-phase systems.

| Enzyme                                            | Enzyme<br>phase | Product<br>K | Product            | Product<br>phase | Product<br>K | Phase<br>components                                                          | Reference                                   |
|---------------------------------------------------|-----------------|--------------|--------------------|------------------|--------------|------------------------------------------------------------------------------|---------------------------------------------|
| Hexokinase+<br>Acetate kinase                     | B               | 0.15<br>0.17 | Glucose-6-P        | T                | -            | PEG 4000<br>PEG-sulfate } 8%(w/w)<br>Dextran T500 }<br>Dextran-ATP } 8%(w/w) | Yamasaki and<br>Suzuki, 1979                |
| Acylase                                           | T               | -            | L-methionine       | B                | 1.54         | PEG 300, 23%(w/w)<br>potassium<br>phosphate, 16%(w/w)                        | Kuhlmann et al.,<br>1980                    |
| Penicillin<br>acylase                             | B               | <0.01        | 6-APA              | T                | 1.35         | PEG 20M, 8.9%(w/w)<br>potassium<br>phosphate, 7.6%(w/w)                      | Paper I                                     |
| Endo- $\beta$ -glucanase+<br>$\beta$ -Glucosidase | B               | 0.16<br>0.14 | Reducing<br>sugars | T                | -            | PEG 20000, 5%(w/w)<br>Crude dextran, 3%(w/w)                                 | Tjerneld et al.,<br>1985a, 1985b,<br>1985c* |

\* Combined with an ultrafiltration unit to recirculate the phase system.

The use of cell organelles for bioconversion has been described (Smeds et al., 1983). Chromatophores from Rhodospirillum rubrum were used to regenerate the coenzyme ATP.

The utilization of living microbial cells for production in aqueous two-phase systems is summarized in Table 5.5. The table also contains the investigations, where both enzymes and cells are used together. Contrary to the values given in the table for the partition coefficient of the products, the production of macromolecular products can be expected to be the most interesting area for production in aqueous two-phase systems. The present results concern only one enzyme, which has an unfavourable K. Similarly, the favourable partition of acetone and butanol was obtained in a phase system holding 25% (w/w) PEG 8000. From a technical point of view this system is unrealistic.

Table 5.5 Microbial production in aqueous two-phase systems.

| Cells                                                        | Cell phase          | Product            | Product recovery phase K | Phase system                                                   | Operation  | Reference                           |
|--------------------------------------------------------------|---------------------|--------------------|--------------------------|----------------------------------------------------------------|------------|-------------------------------------|
| <i>Clostridium tetani</i>                                    | B(T)                | Toxin              | T                        | PEG 4000, 12%(w/v)<br>Dextran D48, 2%(w/v)                     | Continuous | Puziss and Hede <sup>n</sup> , 1965 |
| <i>Saccharomyces cerevisiae</i>                              | B                   | Ethanol            | T                        | PEG 6000, 6%(w/w)<br>Dextran T500, 2%(w/w)                     | Semicont.  | Kühn, 1980                          |
| <i>S. cerevisiae</i> +<br>cellulase+<br>$\beta$ -glucosidase | B                   | Ethanol            | T                        | PEG 8000, 9.6%(w/w)<br>Dextran T40, 3.2%(w/w)                  | Batch      | Hahn-Hägerdal et al., 1981          |
| <i>Clostridium acetobutylicum</i>                            | B                   | Acetone<br>Butanol | T                        | PEG 8000, 25%(w/w)<br>Dextran T40, 6%(w/w)                     | Batch      | Mattiasson et al., 1982             |
| <i>S. cerevisiae</i> +<br>$\alpha$ -amylase                  | B                   | Ethanol            | T                        | PEG 20M, 5%(w/w)<br>Crude dextran, 3%(w/w)                     | Continuous | Larsson and Mattiasson, 1984        |
| <i>Trichoderma reesei</i>                                    | B                   | Cellulase          | T                        | PEG 8000, 5%(w/w)<br>Dextran T500, 7%(w/w)                     | Semicont.  | Persson et al., 1984                |
| <i>Bacillus subtilis</i>                                     | B                   | $\alpha$ -amylase  | T                        | PEG 600, 8%(w/w)<br>PEG 3350, 5%(w/w)<br>Dextran T500, 2%(w/w) | Semicont.  | Paper IV                            |
| <i>B. amylolique-<br/>faciens</i>                            | B( $\rightarrow$ T) | $\alpha$ -amylase  | T                        | -----                                                          | Batch      | Paper V                             |
| <i>B. subtilis</i>                                           | B( $\rightarrow$ T) | $\alpha$ -amylase  | T                        | PEG 3350, 7%(w/w)<br>Dextran T2000, 2.5%(w/w)                  | Semicont.  | Kantelinen et al.                   |

\* Combined with an ultrafiltration unit to recirculate the phase system.



# 6 Recirculation of biocatalysts

The recirculation of biocatalysts is generally considered to have the following advantages: a clean product stream is obtained; substrate is saved for production with cells; high biocatalyst densities are obtained; low amounts of waste material and a continuous process can be designed. However, these advantages have to be discussed against a background, showing how the recirculation influences activity, stability, product concentration, productivity and the cost for the recirculation.

## 6.1 RECIRCULATION OF PENICILLIN ACYLASE

PA is used industrially for the production of 6-APA from natural penicillins. The industrially used PA is recirculated by immobilization to a solid matrix. This makes PA, as well as glucose isomerase, one of the rather few commercially used immobilized enzymes which really has captured an economically acceptable position in an industrial process.

Recirculation of PA has been described with three different methods: (i) immobilization to solid matrices; (ii) an aqueous two-phase system, where PA is partitioned to one phase; and (iii) retention of PA in an ultrafiltration membrane reactor. Data from the literature are summarized in Table 6.1. Differences between the methods will be discussed in the following.

### 6.1.1 Stability and activity

Table 6.1 shows a stability increase of 2-10 times for the systems. This is in the same order of magnitude. However, only the result from the membrane reactor is an operational stability (Greco et al., 1983) and the other values are stor-

Table 6.1. Recirculation of PA.

| Matrix material          | Coupling method                 | Remaining activity increase (%) | Stability increase | Specific productivity ( $\mu\text{mol}/\text{mg}\cdot\text{min}$ ) | Reactor type    | Reference                        |
|--------------------------|---------------------------------|---------------------------------|--------------------|--------------------------------------------------------------------|-----------------|----------------------------------|
| DEAE-cellulose           | 2-amino-4,6-dichloro-s-triazine | 45-81                           | -                  | -                                                                  | -               | Warburton <u>et al.</u> , 1972   |
| Sephadex G-200           | cyanogen bromide                | <48                             | -                  | -                                                                  | CSTR            | Warburton <u>et al.</u> , 1973   |
| crosslinking             | glutaraldehyde                  | -                               | -                  | -                                                                  | STR             | Lagerlöf <u>et al.</u> , 1976    |
| CMC                      | --"--                           | 67-82                           | -                  | 3.9-4.8                                                            | -               | Carleysmith <u>et al.</u> , 1980 |
| Amberlite XAD-7          | --"--                           | 22-90                           | x 2-10             | 0.5-3.5                                                            | -               | Carleysmith and Lilly, 1979      |
| Gelatin                  | --"--                           | -                               | ?                  | -                                                                  | PB (2-stage)    | Park <u>et al.</u> , 1982        |
| crosslinking             | --"--                           | -                               | -                  | -                                                                  | PB with recycle | Haagensen <u>et al.</u> , 1983   |
| polyacrylamide           | -                               | 11                              | x 5.5              | <3.9                                                               | CMR             | Greco <u>et al.</u> , 1983       |
| Aqueous two-phase system | -                               | 70                              | x 3                | 0.65-1.47                                                          | Semi-CSTR       | Paper I+II+III                   |

Reactor types: STR, stirred tank reactor; CSTR, continuous STR; PB, packed bed; CMR, continuous membrane reactor.

age stabilities, which are not really comparable. The remaining activity after immobilization is in the range 22-90%, 70% in the aqueous two-phase system and 11% in membrane reactor.

Little is reported about the stability of the immobilized PA preparations. Industrially used preparations can be expected to have increased stability compared to the free enzyme. However, this is not a general phenomenon. In the immobilization procedure to the matrix material, the coupling chemicals partly destroy the enzyme activity. Part of the enzyme also becomes immobilized in the interior of the matrix beads, where the activity can not be used. One method describes the diffusion of the enzyme into the matrix (Amberlite XAD-7), where it is coupled (Carleysmith et al., 1980). This method gives no "useless" enzyme in the interior of the beads, which in the most optimal case results in a remaining activity of 90%.

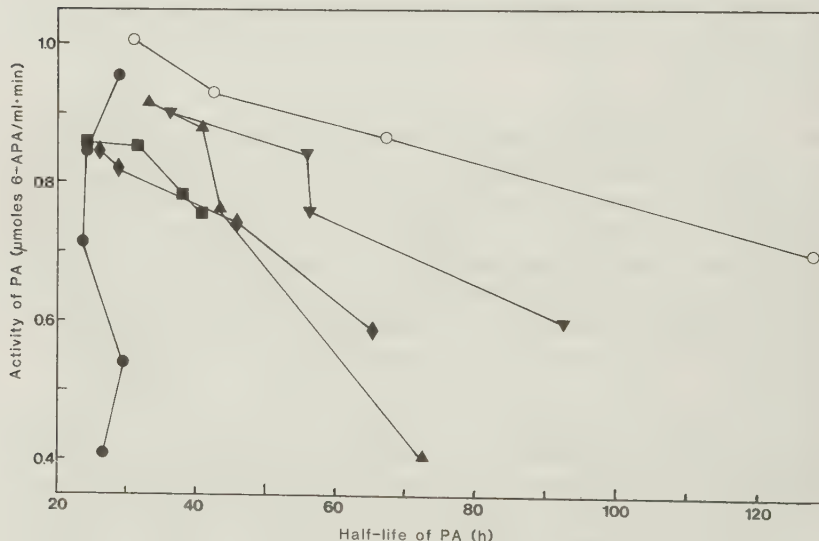
When PA is recirculated in the bottom phase of an aqueous two-phase system composed of PEG and potassium phosphate (Paper I), the enzyme is exposed to a high concentration of potassium phosphate. The half-life of the enzymatic activity in the presence of phosphate can be expected to be increased about three times. Concomitantly, 30% of the enzymatic activity was lost. Thus, an increased biocatalyst stability is obtained at the expense of a lower activity (Figure 6.1).

Recirculation of PA has also been carried out using an unstirred ultrafiltration membrane reactor (Greco et al., 1983), where the enzyme preparation was stabilized within the reactor by addition of a polyacrylamide polymer. The addition of the polymer increased the stability five times and decreased the activity 89%. This was solely ascribed as an effect of polymer-protein interactions.

Principally, polymers can influence the enzyme in two ways: (i) direct interaction between polymer and PA; and (ii) interaction between polymer and the medium water. The action of PEG on PA has been investigated (Paper II+III) and can illustrate this action.

Calorimetry studies indicate a direct interaction between PEG and the protein molecule (Paper II). In protein purification studies including PEG, the proteins behave differently after contact with PEG, also indicating direct contact between PEG and the protein surface (Tony Atkinson, personal communication).

The interaction between PEG and the surrounding water focuses on the three primary functions of water for enzymes: (i) enzyme conformation (Zaks and Klibanov, 1984); (ii) reactant, e.g. in lipolysis (Andersson, 1980); and (iii) diffusion medium (Acker, 1969).



**Figure 6.1.** Activity of PA as a function of half-life of enzymatic activity. Half-life data from Paper II. PEG 600 (●—●), PEG 1550 (▲—▲), PEG 3350 (◆—◆), PEG 8000 (▼—▼), PEG 20M (■—■) and potassium phosphate (○—○). (from Paper III).

The water available was measured as the water activity and decreased in the PEG solutions (Paper II). Differential scanning calorimetry (DSC) measurements also showed that PEG had an unusually high ability to associate water (Blow et al.,1978; Tilcock and Fisher,1982).

The stability and activity of PA in relation to the water activity were investigated in PEG solutions (Paper II+III). No relation was found for the stability, but a relation was found between the enzymatic activity and the water activity. An increased stability of PA in PEG solutions seems instead to be due to a direct interaction between the polymer and the enzyme (Figure 6.1).

Increased molecular weight and increased concentration both favoured an increased half-life of PA. Thus, PEG might be an alternative to polyacrylamides for use in membrane reactors to increase the stability of PA. A 30% (w/w) PEG 8000 solution in a stirred membrane reactor could increase the stability three times and decrease the activity 30% (Figure 6.1).

### 6.1.2 Productivity

Data for the different systems are in the same range, 0.5-4.8  $\mu\text{mole/mg}\cdot\text{min}$  (Table 6.1). For immobilized PA few specific productivity data are available, but the specific productivity under assay conditions have been estimated from immobilization data. The specific productivity estimations in the membrane reactor and in the aqueous two-phase systems were obtained under operational conditions. The results are also very dependent on the protein content in the enzyme preparation, i.e. a purified enzyme gives a higher value, which also makes it hard to do a fair comparison.

### 6.1.3 Reactor design

The reactor design is important for the production with a recirculated biocatalyst. The deacylation reaction of PA produces acid, which has to be titrated. The immobilized enzyme preparations can be used both in stirred tank reac-

tors, continuously or as repeated batches, and in packed beds (Table 6.1). The pH control is direct in the stirred tanks, but in packed bed reactors pH control is performed outside the reactor. High flow rates through a series of beds can be used with low conversion in each step and pH adjustment between the reactors.

Semicontinuous conversion has been described in aqueous two-phase systems (Paper I), where the top phase was replaced in every step with a new top phase and fresh substrate (Figure 6.2). Although the phase system contained a high concentra-

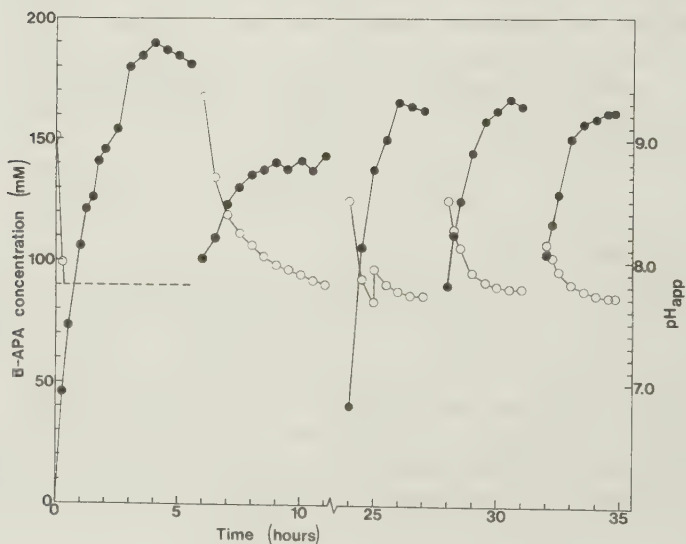


Figure 6.2 Repeated batch conversion of BP, 10%(w/v), to 6-APA with PA at 37°C. The BP addition as calculated on the total phase volume for the first conversion and on the changed volume for the following conversions. After 24 hours was added to re-establish the original activity. 6-APA concentration (●) and apparent pH (○) are indicated. (from Paper I).

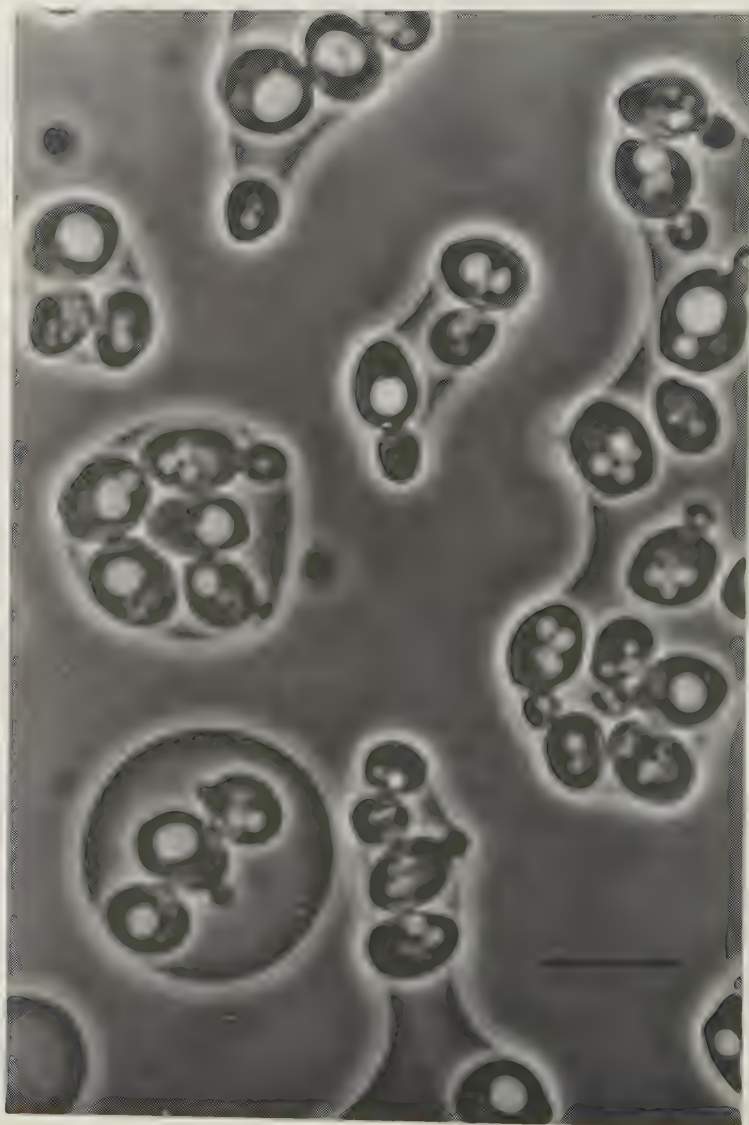


tion of potassium phosphate, titration was necessary to diminish the pH drop. This phase system was sensitive, and changed its composition when pH and temperature differed. The composition of the fresh top phase thus has to be identical to the removed top phase. This was not achieved, since the exact composition was not known. It was thus observed, that the 6-APA concentration increased at the start of every conversion. This was due to a decreased volume ratio ( $V_T/V_B$ ) resulting in replacement of smaller volumes of top phase for every step. This can partly be overcome by recirculating the top phase after 6-APA has been removed. Aqueous two-phase systems offer a possibility to use a continuous process in a mixer-settler type reactor, which has partly been described for cellulose hydrolysis (Tjerneld et al., 1985b, 1985c).

#### 6.1.4 Mass transfer

The biocatalyst performance is also important for the production with a recirculated biocatalyst. For PA, the transport of substrate to and the removal of the product from the catalytical site of the enzyme is important, especially as the enzyme is product inhibited (Balasingham et al., 1972). Mass transfer limitations can be expected to occur in an immobilized biocatalyst due to the dense structure of the catalyst (White and Dorion, 1961). For aqueous two-phase systems this problem is small, as the enzyme is free in suspension, although partitioned to only one of the phases, and a droplet size with a diameter less than 10  $\mu\text{m}$  is obtained (Figure 6.3). Thus, the droplet size is much smaller than the size of the immobilization beads. The small droplet size gives a large interfacial area between the phases, which is advantageous for the mass transfer.

Figure 6.3 The droplet size in an aqueous two-phase system compared to the size of Saccharomyces cerevisiae cells. Scale bar, 10  $\mu$ m.



## 6.2 RECIRCULATION OF BACILLUS SP.

The  $\alpha$ -amylase production with recirculated Bacillus sp. is summarized in Table 6.2.

### 6.2.1 Cell-free product streams?

One objective for the use of immobilization and aqueous two-phase systems is to obtain cell-free product streams. However, for  $\alpha$ -amylase production with B. amyloliquefaciens immobilized in  $\kappa$ -carrageenan (Shinmyo et al.,1982), the cells grew out of the gel. This is also a generally observed phenomenon for the commonly used system with immobilized yeast cells in calcium alginate. Thus, the gel is the place for the product formation, and it further acts as a seed for the cells in the surrounding medium, which are often very productive. Aqueous two-phase systems can give cell-free product streams, however, depending on the size and the surface properties of the cells as has been discussed previously in Chapter 5.2.4.

### 6.2.2 Substrate

A large part of the substrate in a batch process with living cells is used only to propagate the cell population in the reactor. Recirculation diminishes this demand, thus decreasing the substrate use. This was shown in a process with semicontinuous production in an aqueous two-phase system, where 6% of the substrate was saved (Paper IV).

Natural substrates for production with whole living cells often contain insoluble particles. Such substrates, e.g. particulate starch, was used for production in aqueous two-phase systems (Paper IV; Larsson and Mattiasson,1984). However, when immobilized cells are used, these substrates can not be used, due to the sterical hindrance in the gel. This led Kokubu et al.(1978) to develop a medium composed of meat extract and yeast extract, for the production of  $\alpha$ -amylase.

Table 6.2 Production of  $\alpha$ -amylase.

| Cells                             | Immobilization        | Phase system * | Product conc. ** | Productivity ** | Production type *** | Reference                    |
|-----------------------------------|-----------------------|----------------|------------------|-----------------|---------------------|------------------------------|
| <i>Bacillus subtilis</i>          | -                     | -              | 100              | 30              | B                   | Kokubu <u>et al.</u> , 1978  |
|                                   | polyacrylamide        | -              | 13               | 26              | Semi-C              |                              |
| <i>Bacillus amyloliquefaciens</i> | -                     | -              | 80               | 50              | B                   | Shinmyo <u>et al.</u> , 1982 |
|                                   | $\kappa$ -carrageenan | -              | 3.1              | 45-62           | C                   |                              |
| <i>Bacillus subtilis</i>          | -                     | -              | 58               | 8.4             | B                   | Paper IV                     |
|                                   |                       | A              | 85-135           | 7.7-16.9        | Semi-C              |                              |
|                                   |                       | B              | 35               | 5.3             | B                   |                              |
| <i>Bacillus amyloliquefaciens</i> | -                     | -              | 60               | 21              | B                   | Paper V                      |
|                                   |                       | A              | 38               | 5               | B                   |                              |
|                                   |                       | B              | 42               | 10              | B                   |                              |
| <i>Bacillus subtilis</i>          | -                     | -              | 16               | 44              | B                   | Kantelininen <u>et al.</u>   |
|                                   |                       | C              | 15-18            | 20-32           | Semi-C              |                              |

\* Phase system: A; PEG 600, 8% (w/w), PEG 3350, 5% (w/w), Dextran T500, 2% (w/w)  
 B; PEG 3350, 9% (w/w), Dextran T500, 2% (w/w)  
 C; PEG 3350, 7% (w/w), Dextran T2000, 2.5% (w/w)

\*\* relative values, which can not be compared outside each investigation.

\*\*\* B; batch, C; continuous.

### 6.2.3 Cell density

High cell densities can be obtained with immobilized cells. These high densities can only be valuable if the activity of all the cells is used. However, it has been observed that mainly the cells in the bead surface are active (Shinmyo et al.,1982). In aqueous two-phase systems, the cells are mainly collected in one of the phases. When mixed, the phase system becomes an emulsion of very small droplets in which one can expect all the cells to have access to the substrate. In a semicontinuous production over several steps, cells and fragments from dead cells can accumulate in the bottom phase, which is a disadvantage.

### 6.2.4 Waste material

The amount of waste material is decreased by recirculating the cells. This can be visualized by an example from the commercial production of ethanol (Alfa-Laval,1983). In a normal batch process 13 liters of draff was produced for every liter of ethanol. In a process where the cells were recirculated by centrifugation and also together with other unit operations, only 0.7 liter draff per liter of ethanol was produced. This decreased the waste problem drastically.

When aqueous two-phase systems are used, polymers are added to the solution. These polymers increase the waste material obtained with the product stream. This can be overcome by recirculation of the polymers after extraction of the product to a salt phase, which together with the polymers forms a phase system (Kula et al.,1982), or by recirculating the polymers from the product stream with ultrafiltration (Larson and Mattiasson,1984; Tjerneld et al.,1985c).

### 6.2.5 Recirculation systems in relation to product concentration

The systems for recirculation of Bacillus sp., which are summarized in Table 6.2 make continuous and semicontinuous operation possible. Continuous operation gives the advantage of a steady product flow, giving a better use of the equipment available. It is impossible to compare the results

concerning the product concentrations and productivities from different investigations, as all investigators have their own analytical method. Thus, the results can only be compared within the same investigation. Furthermore, the figures in Table 6.2 are adjusted to make them easier to read.

During continuous operation with immobilized B. amyloliquefaciens cells, only 4% of the product concentration was obtained, compared to a batch fermentation (Shinmyo et al.,1982). In another study with semicontinuous  $\alpha$ -amylase production with immobilized B. subtilis cells, Kokubu et al.(1978) reported a concentration which was only 13% of the concentration in a comparable submerged culture. Despite the low product concentrations, the productivities were unaltered. The low product concentration is followed by an increased water content in the product flow, which makes it expensive to concentrate the product.

Continuous production with an aqueous two-phase system has been described by Puziss and Hede'n (1965). However, they used a continuous flow with a total mixed phase system. Aqueous two-phase systems also offers a possibility to use the bottom phase as a stationary phase with biocatalyst, and to use the top phase as a mobile phase for substrate addition and product removal. This has been studied for production of ethanol (Larsson and Mattiasson,1984) and glucose and xylose (Tjerneld et al.,1985b,1985c) in mixer-settler type reactors.

Semicontinuous production offers another possibility to use aqueous two-phase systems. This has been investigated for  $\alpha$ -amylase production (Paper IV; Kantelinen et al.) and cellulase production (Persson et al.,1984). A product concentration can be obtained in the product phase (the top phase), which is in the same range as the concentration obtained in a batch process.

In one three-step fermentation with a strain of Bacillus subtilis in an aqueous two-phase system, higher product concentrations were obtained (Paper IV). This could be due to an extraction effect. However, in another phase system, the production was repressed (Paper IV). Furthermore, when the



production of  $\alpha$ -amylase was studied with a B. amyloliquefaciens strain in the same two phase systems (Paper V), the amylase production was repressed in both phase systems (Paper V) and the productivity was only one fourth and half, respectively, as compared to the reference fermentation. Thus, an increased enzyme production in aqueous two-phase systems is not a general phenomenon among strains of Bacillus. The increased product concentrations in certain phase systems with certain strains indicate an effect of the phase systems on the cells rather than a product extraction effect.

#### 6.2.6 Influence of the medium

Ramgren et al. could show that the increased production of  $\alpha$ -amylase was exclusively an effect of PEG 600 in the solution. PEG can be expected to influence several factors both in the medium and directly with the cells.

PEG reduces the water activity of the medium in these solutions, although to a minor extent (Paper II+V). It has been proposed that the decreased water activity affects the kinetics and energetics of bacterial growth (Esener et al., 1981), leading to increased energy demands for the maintenance function, which then would decrease the enzyme production. The prolonged periods of high metabolic activity, indicated as the dissolved oxygen, obtained in aqueous two-phase systems and PEG solutions (Paper V) might be explained in terms of a physiological stress for the cells leading to higher demands for the maintenance function.

The oxygen transfer rate,  $OTR = k_L \cdot a \cdot (c^* - c_L)$ , can in several ways be expected to be influenced by aqueous two-phase systems and PEG solutions. Thus, the surface tension is decreased and in the bottom phase of the phase system, the viscosity is increased (Anneli Svensson, personal communication). The polarity is also decreased in PEG solutions (Arnold et al., 1985). The total influence of these different parameters affecting the oxygen transfer rate is difficult to predict without further investigations.

### 6.2.7 Influence of PEG

PEG is often used as a fusogen. Protoplasts of cells are used in fusion. The literature about fusion is extensive (for a review, see Fisher and Goodall, 1981). One might speculate that PEG has a similar influence on cells during a fermentation as it has on protoplasts during fusion. However, it must be remembered that the bacterial cells have a cell wall, while the protoplasts only have parts of the cell wall left.

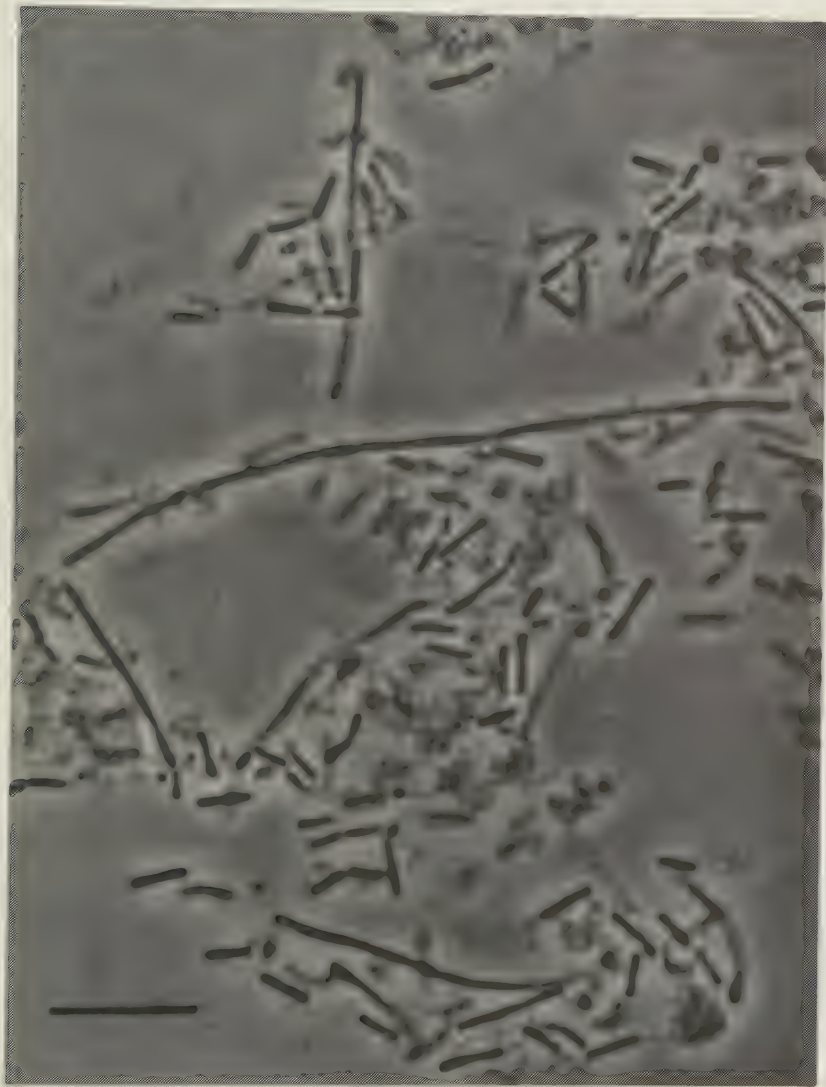
Fusion consists of two processes: (i) bringing the protoplasts in close proximity; and (ii) fusion.

Exclusion of surrounding water brings the protoplasts in close proximity due to a steric exclusion mechanism. PEG is assumed to bind water molecules both to the terminal hydroxyl moieties and to the ether-linked oxygen atoms in the polymers (Blow et al., 1978, Tilcock and Fisher, 1982).

Indirect evidence exists for PEG disordering the membrane structure: PEG is used for transformation of plasmids into protoplasts (Chang and Cohen, 1979); PEG releases membrane constituents from HEP-2 cells (McCammon and Fan, 1979); PEG makes the membranes abnormally permeable to ions and small molecules (Aldwinckle et al., 1982); PEG extracts water from the membrane bilayers (Arnold et al., 1983), PEG decreases the dielectric constant (Arnold et al., 1985); and PEG makes plant protoplasts leak intracellular cell components (Hahn-Hägerdal et al.).

The disordered cell membranes might be the reason, why morphologically different B. amyloliquefaciens cells occur during growth in a PEG medium (Figure 6.4). Furthermore, Ramgren et al. also found morphologically similar cells of B. subtilis in a PEG medium. The disordered cell membranes might obstruct the cell replication, instead swelling the cells as the metabolism proceeds. It can be argued that the bacterial cells, with the cell wall, ought to be more resistant to PEG treatment than the protoplasts. An analysis of the cell content and the cell wall composition might give an insight into the reason for this morphological abnormality.

**Figure 6.4** Bacillus amyloliquefaciens cells from a fermentation in a 20% (w/w) PEG 600 medium. Scale bar, 10  $\mu\text{m}$ .



### 6.2.8 Membrane effects

PEG reduces the dielectric constant of the medium (Arnold et al.,1985). The formation of a membrane bilayer demands a minimum value of the dielectric constant. The increased cation permeability of cell membranes (Aldwinckle et al.,1982) can be explained as an effect of a changed dielectric constant, lowering the energy to transfer a charge into the membrane (Arnold et al.,1985).

Proton motive force is essential to the translocation of polypeptides through membranes in some but not all membrane systems. It was recently reported, that B. amyloliquefaciens requires an electrochemical membrane potential to secrete  $\alpha$ -amylase (Mure'n and Randall,1985). An increased permeability of the cell membranes to ions (Arnold et al.,1985) thus counteracts the cells ability to maintain an electrochemical gradient across the membrane. When the electrochemical gradient was dissipated by uncouplers, cells of B. amyloliquefaciens accumulated a precursor form of  $\alpha$ -amylase in the cell membranes (Mure'n and Randall,1985). A high amount of a precursor form of  $\alpha$ -amylase, accumulated in the cellular membranes, might cause the cells to swell (Figure 6.4) and change the surface properties of the cells (Chapter 5.2.4; Paper V).

The cells of B. subtilis became more hydrophilic and increased the enzyme production in a PEG 600 medium (Chapter 5.2.4; Paper V). Contrary, the cells of B. amyloliquefaciens became more hydrophobic and the enzyme production was repressed (Chapter 5.2.4; Paper V). No conclusions can be made concerning the relation between the cell surface properties and the enzyme production. However, it can be concluded that the cell membranes and/or cell walls are different for the two strains of B. subtilis and B. amyloliquefaciens.

# 7 Conclusions

Recirculation in aqueous two-phase systems gives a possibility to "bind" an enzyme in one phase, although the enzyme is still free in suspension. However, when the system is used for semicontinuous or continuous production with replacement of the other phase, even a small partition of the enzyme to this phase can wash the enzyme out of the reactor. This problem has been overcome by using a membrane unit together with the phase system.

Recirculation of living cells in aqueous two-phase systems has the advantage that it is easier to obtain a onesided partition but this is not a general phenomenon for cells. A small leakage of cells can be accepted during recirculation of cells, as the cell population regenerates itself. However, the cells then pollute the product stream.

The environment in the phase system influences the enzyme stability and activity. The phase systems also change the surface properties of cells.

The really interesting area, both for research and biotechnology, is the production of macromolecular extracellular products with living microorganisms in aqueous two-phase systems. (i) The physiology of the cell surfaces can be expected to be sensitive to environmental conditions, when an extracellular macromolecule is produced. (ii) Macromolecules are often easier to partition away from the cells. (iii) Macromolecular products often have a higher price, which is necessary to pay the costs of the phase systems in a biotechnological process.

As mentioned above, the costs of the phase systems is a limiting factor for the technical exploitation of aqueous two-phase systems. The high costs come almost exclusively

from the price of purified dextrans. For protein purification purposes, this problem has been overcome by using PEG-salt systems. These systems can not be used together with living cells. Promising results have recently been reported with a starch-based polymer, that can replace the dextran (Tjerneld et al., 1986). However, for a microorganism producing a starch degrading enzyme, this polymer can not be used.

FELIX QUI POTUIT COGNOSCERE CAUSAS!



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# References

- Acker LW (1969) Food Technol 23:27-40
- Albertsson P-Å (1971) Partition of Cell Particles and Macromolecules, 2nd ed, Almqvist & Wiksell, Uppsala
- Albertsson P-Å (1977) Endeavour 1:69-74
- Aldwinckle TJ, Ahkong QF, Bangham AD, Fisher D, Lucy JA (1982) Biochim Biophys Acta 689:548-560
- Alfa-Laval (1983) In "Minne från framtiden", pp 12-14
- Andersson RE (1980) Appl Environ Microbiol 39:36-40
- Arnold K, Pratsch L, Gawrisch K (1983) Biochim Biophys Acta 728:121-128
- Arnold K, Herrmann A, Pratsch L, Gawrisch K (1985) Biochim Biophys Acta 815:515-518
- Balasingham K, Warburton D, Dunnill P, Lilly MD (1972) Biochim Biophys Acta 276:250-256
- Bergey's Manual of Determinative Bacteriology (1974) 8th ed, Williams & Wilkins Co, Baltimore
- Beijerinck MW (1896) Zentralbl Bacteriol 2:627,698
- Blow AMJ, Botham GM, Fisher D, Goodall AH, Tilcock CPS, Lucy JA (1978) FEBS Letters 94:305-310
- Carleymith SW, Lilly MD (1979) Biotechnol Bioeng 21:1057-1073
- Carleymith SW, Dunnill P, Lilly MD (1980) Biotechnol Bioeng 22:735-756
- Chang S, Cohen SN (1979) Molec Gen Genet 168:111-115
- Cole M (1967) Process Biochem 2:35-46
- Cole M (1969a) Biochem J 115:733-739
- Cole M (1969b) Biochem J 115:747-756
- Cole M (1969c) Biochem J 115:757-764
- Davis BD, Tai P-C (1980) Nature 283:433-438
- Esener AA, Bol G, Kossen NWF, Roels JA (1981) In Advances in Biotechnology I (Moo-Young M, Robinson CW, Vezina C, Eds) Pergamon Press, Toronto, pp 339-344
- Fisher D, Goodall AH (1981) Techniques Cell Physiol P115:1-36
- Fogerty WM (1983) In Microbial Enzymes and Biotechnology (Fogerty WM, Ed) Applied Science Publishers, London, New York, pp 1-92

Gerson DF (1980) Biochim Biophys Acta 602:269-280

Gerson DF, Akit J (1980) Biochim Biophys Acta 602:281-284

Godfrey T (1983) In Industrial Enzymology: The Application of Enzymes in Industry (Godfrey T, Reichelt J, Eds) The Nature Press, New York, pp 437-449

Greco Jr G, Veronese F, Largajolli R, Gianfreda L (1983) Eur J Appl Microbiol Biotechnol 18:333-338

Haagensen P, Karlsen LG, Petersen J, Villadsen J (1983) Biotechnol Bioeng 25:1873-1895

Hahn-Hägerdal B, Mattiasson B, Albertsson P-Å (1981) Biotechnol Lett 3:53-58

Hahn-Hägerdal B, Hosono K, Zachrisson A, Bornman CH (Submitted for publication)

Kantelinen A, Poutanen K, Linko M, Markkanen P In Enzyme Engineering 8 (submitted for publication)

Kasche V, Haufler U, Zöllner R (1984a) Hoppe-Seyler's Z Physiol Chem 365:1435-1443

Kasche V, Haufler U, Riechmann L (1984b) In Enzyme Engineering 7 (Laskin AI, Tsao GT, Wingard LB Jr, Eds) Ann N Y Acad Sci 434:99-105

Kasche V (1986) Enzyme Microb Technol 8:4-16

Klesov AA, Galaev IY, Shvyadas V-YK (1977) Bioorg Khimiya 3:663-672

Konecny J, Schneider A, Sieber M (1983) Biotechnol Bioeng 25:451-467

Kokubu T, Karube I, Suzuki S (1978) Eur J Appl Microbiol Biotechnol 5:233-240

Kroner KH, Hustedt H, Kula M-R (1982) Biotechnol Bioeng 24:1015-1045

Kroner KH, Hustedt H, Kula M-R (1984) Process Biochem 19:170-179

Kuhlmann W, Halwachs W, Schügerl K (1980) Chem Ing Tech 52:607, Synopse 821

Kula M-R, Kroner KH, Hustedt H (1982) In Advances of Biochemical Engineering (Fiechter A, Ed) Vol 24, Springer-Verlag, pp 73-118

Kutzbach C, Rauenbusch E (1974) Hoppe-Seyler's Z Physiol Chem 355:45-53

Kühn I (1980) Biotechnol Bioeng 22:2393-2398

Lagerlöf E, Nathorst-Westfeldt L, Ekström B, Sjöberg B (1976) In Immobilized Enzymes (Mosbach K, Ed) Methods Enzymol 44:759-768

Larsson M, Mattiasson B (1984) In Enzyme Engineering 7 (Laskin AI, Tsao GT, Wingard LB Jr, Eds) Ann N Y Acad Sci 434:144-147

Leuchtenberger W, Karrenbauer M, Plöcker U (1984) In Enzyme Engineering 7 (Laskin AI, Tsao GT, Wingard LB Jr, Eds) Ann N Y Acad Sci 434:78-86

Mattiasson B, Ling TGI (1980) J Immunol Methods 38:217-223

Mattiasson B, Ling TGI, Ramstorp M (1981) J Immunol Methods 41:105-114

Mattiasson B, Souminen M, Andersson E, Häggström L, Albertsson P-Å, Hahn-Hägerdal B (1982) In Enzyme Engineering 6 (Chibata I, Fukui S, Wingard LB Jr, Eds) Plenum Publishing Corporation, pp 153-155

McCammon JR, Fan SCF (1979) Biochim Biophys Acta 551:67-73

Methods of Enzymology: Immobilized Enzymes (1976)(Mosbach K, Ed) Vol 44, Academic Press, New York

Mure'n EM, Randall LL (1985) J Bacteriol 164:712-716

Nagashima M, Azuma M, Nogushi S, Inuzuka K, Samejima H (1984) Biotechnol Bioeng 26:992-997

Park JM, Choi CY, Seong BL, Han MH (1982) Biotechnol Bioeng 24:1623-1637

Persson I, Tjerneld F, Hahn-Hägerdal B (1984) Enzyme Microb Technol 6:415-418

Priest FG (1984) Extracellular Enzymes, Aspects of Microbiology 9, Van Nostrand Reinhold (UK) Co Ltd, Wokingham, England

Puziss M, Hede'n C-G (1965) Biotechnol Bioeng 7:355-366

Ramgren MIM, Andersson E, Hahn-Hägerdal B (manuscript in preparation)

Randall LL, Hardy SJS (1984) Microbiol Rev 48:290-298

Sakagushi K, Murao S (1950) J Agric Chem Soc Japan 23:411

Schürch S, Gerson DF, McIvers DJL (1981) Biochim Biophys Acta 640:557-571

Shaw DJ (1970) Introduction to Colloid and Surface Chemistry, 2nd ed, Butterworths, London-Boston

Shinmyo A, Kimura H, Okada H (1982) Eur J Appl Microbiol Biotechnol 14:7-12

- Smeds A-L, Veide A, Enfors S-O (1983) Enzyme Microb Technol 5:33-36
- Svedas VK, Margolin AL, Borisov IL, Berezin IV (1980) Enzyme Microb Technol 2:313-317
- Tilcock CPS, Fisher D (1982) Biochim Biophys Acta 688:645-652
- Tjerneld F, Persson I, Albertsson P-Å, Hahn-Hägerdal B (1985a) Biotechnol Bioeng 27:1036-1043
- Tjerneld F, Persson I, Albertsson P-Å, Hahn-Hägerdal B (1985b) Biotechnol Bioeng 27:1044-1050
- Tjerneld F, Persson I, Albertsson P-Å, Hahn-Hägerdal B (1985c) Biotechnol Bioeng Symp 15 (in press)
- Tjerneld F, Berner S, Cajarville A, Johansson G (1986) Enzyme Microb Technol (in press)
- Vandamme EJ (1977) Adv Appl Microbiol 21:89-123
- Veide A, Smeds A-L, Enfors S-O (1983) Biotechnol Bioeng 25:1789-1800
- Warburton D, Balasingham K, Dunnill P, Lilly MD (1972) Biochim Biophys Acta 284:278-284
- Warburton D, Dunnill P, Lilly MD (1973) Biotechnol Bioeng 15:13-25
- White ML, Dorion GH (1961) J Polymer Sci 55:731-740
- Zaks A, Klibanov AM (1984) Science 224:1249-1251
- Yamazaki Y, Suzuki H (1979) Rep Ferment Res Inst Japan 52:33-40



# I Enzymatic conversion in aqueous two-phase systems: deacylation of benzylpenicillin to 6-aminopenicillanic acid with penicillin acylase

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*The conversion of benzylpenicillin (BP) to 6-aminopenicillanic acid (6-APA) using penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) has been studied in aqueous two-phase systems. In a system composed of 8.9% (w/w) PEG 20000/7.6% (w/w) potassium phosphate the enzyme was almost completely partitioned to the bottom phase ( $K < 0.01$ ), which allowed repeated batch conversions, recirculating the enzyme several times. The initial specific productivities were 0.31–1.47  $\mu\text{mol}$  6-APA  $\text{mg protein}^{-1} \text{min}^{-1}$  in repeated conversions over five steps. The yield obtained from the top phase was 0.47–0.71  $\text{mol}$  6-APA  $\text{mol BP}^{-1}$ . The results are discussed in relation to recirculating the enzyme by immobilizing it to a solid matrix. Despite the high phosphate concentration in the bottom phase the system needs to be titrated in order for the reaction to proceed. Titration of the top phase alone protected the enzyme from denaturation by strong alkali used for the titration.*

**Keywords:** Antibiotics; penicillin acylase; benzylpenicillin; 6-aminopenicillanic acid; enzymatic conversion; aqueous two-phase systems

## Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) from *Escherichia coli* catalyses the deacylation of benzylpenicillin (BP) to 6-aminopenicillanic acid (6-APA), which is used for the industrial production of semisynthetic penicillins. The reaction is a pH-dependent equilibrium which is shifted towards synthesis of BP when the pH decreases. The optimum pH for production of 6-APA is 7.5–8.0. The formation of 6-APA is, however, accompanied by formation of phenylacetic acid, which decreases the pH. To shift the equilibrium in favour of 6-APA formation, it is important that the products are removed from the enzyme.

To be able to reuse the enzyme several times, penicillin acylase used for industrial deacylation today is immobilized on to solid matrices and several studies have been reported with different matrix materials.<sup>1–4</sup> The immobilization results in diffusional limitations within the matrix which cause an accumulation of products and a concomitant decrease in productivity.

Another method to recirculate an enzyme is to enrich the enzyme in one phase in an aqueous two-phase system. Aqueous two-phase systems are formed when solutions of two water-soluble polymers are mixed.<sup>5</sup> They can also be formed by a water-soluble polymer solution and a salt solution. Aqueous two-phase systems have very low interfacial tension (0.0001–0.1  $\text{dyn cm}^{-1}$ ) between the phases. When the phases are stirred, a very large interfacial area is

formed, which promotes good mass transfer and avoids diffusional limitations.

Aqueous two-phase systems were described a long time ago,<sup>6</sup> but there has been little technical interest in their use. Recently, the use of these systems for the large-scale isolation and purification of intracellular enzymes has been reviewed.<sup>7–8</sup> They have also been applied to biological analysis<sup>9</sup> and cofactor recirculation.<sup>10–11</sup> Bioconversions in aqueous two-phase systems have so far only been demonstrated on a laboratory scale in the context of toxin production by *Clostridium tetani*,<sup>12</sup> conversion of cellulose<sup>13</sup> and starch<sup>14</sup> to ethanol, and the production of acetone and butanol using *Clostridium acetobutylicum*.<sup>15</sup>

The present study investigated the possibility of using an aqueous two-phase system for recirculating the enzyme penicillin acylase in the deacylation of benzylpenicillin.

## Materials

Penicillin acylase (10.4 U  $\text{mg protein}^{-1}$ ; assay described below) and the potassium salt of benzylpenicillin (BP) (90% purity compared to the BP used for assays) for the conversion studies were generous gifts from Astra AB, Södertälje, Sweden. The potassium salt of BP for assays was obtained from Calbiochem, San Diego, CA, USA, and 6-aminopenicillanic acid (6-APA) from Sigma Chemical Co., St Louis, MO, USA. Dextran T 40 and T 10 were purchased from Pharmacia Fine Chemicals, Uppsala.

Sweden. Three different types of poly(ethylene glycol) (PEG) were used. Carbowax PEG 8000 (MW 7000–9000) and PEG 3350 (MW 3000–37000) were obtained from Union Carbide, Danbury, CT, USA, and PEG 20000 (MW 17 000–20 000) was purchased from Serva, Heidelberg, FRG. All other chemicals were reagent grade and obtained from commercial sources.

## Methods

### Preparation of aqueous two-phase systems

The preparation and analysis of aqueous two-phase systems has been described by Albertsson.<sup>5</sup> The partition coefficient for the enzyme was defined as  $K$ , where  $K = \text{activity (top phase)}/\text{activity (bottom phase)}$ . Concentration was used instead of activity for the determination of partition coefficient for substrate and products. The dextran used for the preparation of phase systems was assumed to be water-free. For pH measurement in the phase systems, three different electrodes were tested in the same phase system (pH 7.8), giving different (and too low) pH values. These varied between pH 0.1 and 0.2.

We therefore used the following method for pH determination. The absorbance of 2-cresolsulphonethalein (Cresol Red) was measured at 573 and 435 nm. A plot of  $\log_{10}(A_{573}/A_{435})$  versus pH gave a linear relationship between pH 7.0 and 8.8. This method was used for pH determination during the PA partition studies. However, electrodes had to be used to follow the pH during the reaction and in this case pH is given as apparent pH.

### Analysis

BP was extracted and analysed by the method described by Cole.<sup>16</sup> Phenylacetic acid was determined by measuring the absorbance at 258 nm. 6-APA was analysed by the method described by Balasingham *et al.*<sup>17</sup> in which 4-*N,N*-dimethylaminobenzaldehyde reacts with 6-APA to form a coloured Schiff's base which can be measured at 415 nm. The protein content of the enzyme solution was determined by the Folin method of Lowry *et al.*<sup>18</sup>

The composition of the top phase was determined from the phase diagram according to the method described by Albertsson.<sup>5</sup> As the phase diagram is pH and temperature

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dependent, the determination was modified with dry weight determinations at 105°C overnight.

Enzyme activity was determined according to the method of Balasingham *et al.*<sup>17</sup> using a 10 mg ml<sup>-1</sup> (27 mM) solution of BP in 0.05 M sodium phosphate buffer pH 7.8. One unit of enzyme activity was defined as the amount of enzyme required to produce one micromole of 6-APA per minute.

### Batch conversion

Batch conversion were carried out in 200 ml Erlenmeyer flasks with 50 ml reaction solution in a thermostatically controlled water bath. The stirring was performed at 250 rev/min with a 3 cm magnetic bar with water resistant electromagnetic driving units from Medelco AB, Hågersten, Sweden. The reaction was followed by measuring the formation of 6-APA.

## Results and discussion

### Partition of penicillin acylase, BP, 6-APA and phenylacetic acid

Recirculation of the enzyme in aqueous two-phase systems requires that no enzyme is lost when the product is removed, i.e. the enzyme should be partitioned to the bottom phase and be stable there. Ideally, the substrate partitions to the same phase as the enzyme and the product partitions to the opposite phase. However, the partition and stability of the enzyme are the most important objectives.

To partition penicillin acylase to the bottom phase means that the partition coefficient,  $K$ , should be very small. A theory for partition of protein in aqueous two-phase systems has been proposed and examined.<sup>5,19</sup> This theory demands a thorough knowledge of the physico-chemical properties of the protein. Therefore, the method generally used for screening of suitable aqueous two-phase systems for one-sided partition of a particular protein is the one of trial and error. A large number of phase systems with varying phase components, molecular weights of phase components and salts have been investigated in the present study and a selection of the data is summarized in *Tables 1* and *2*. Partition in PEG–dextran systems with different

**Table 1** Partition of penicillin acylase in aqueous two-phase systems composed of PEG and dextran. All data at pH 7.8 and 22°C.  $K$  is based on activity measurement and is defined as the activity in the top phase divided by the activity in the bottom phase

| PEG type | % (w/w) | Dextran type | % (w/w) | Buffer              | Molarity | $K$  |
|----------|---------|--------------|---------|---------------------|----------|------|
| 8000     | 7.5     | T 40         | 6.0     | Tris                | 0.5      | 0.14 |
| 8000     | 7.5     | T 40         | 6.0     | Tris                | 0.3      | 0.13 |
| 3000     | 7.5     | T 40         | 6.0     | Sodium phosphate    | 0.2      | 0.10 |
| 8000     | 7.5     | T 40         | 6.0     | Sodium phosphate    | 0.5      | 0.04 |
| 8000     | 7.5     | T 40         | 6.0     | Potassium phosphate | 0.5      | 0.35 |
| 8000     | 7.5     | T 40         | 6.0     | Sodium phosphate    | 0.05     | 0.15 |
| 20000    | 10.0    | T 10         | 5.0     | Sodium phosphate    | 0.12     | 0.03 |

**Table 2** Partition of penicillin acylase in aqueous two-phase systems composed of PEG and salt. The experimental conditions are the same as in *Table 1* except for phase system asterisked, where the temperature was 37°C. This phase system is formed at a temperature of ~25°C

| PEG type | % (w/w) | Salt type           | % (w/w) | Phase volume ratio (top/bottom) | $K$       |
|----------|---------|---------------------|---------|---------------------------------|-----------|
| 8000     | 10.0    | Potassium phosphate | 10.0    | 0.69                            | 0.02–0.03 |
| 20000    | 10.0    | Potassium phosphate | 10.0    | 0.64                            | 0.02–0.03 |
| *20000   | 8.9     | Potassium phosphate | 7.6     | 0.88                            | <0.01     |
| 3350     | 12.0    | Magnesium sulphate  | 10.0    | 0.43                            | <0.01     |

molecular weights of polymer and with various salts is summarized in Table 1. A combination of high molecular weight PEG and low molecular weight dextran gave the lowest  $K$  value for penicillin acylase. The lowest  $K$  was found when 0.5 M sodium phosphate buffer was used with the polymers. This is probably due to the fact that in this system phosphate acts as both a buffering agent and a phase forming component. We therefore continued to study the partition of penicillin acylase in PEG-salt systems (Table 2).

A common property of the PEG-salt phase systems investigated is that the bottom phase contains more water than the top phase, which is opposite to the PEG-dextran systems.<sup>5</sup> This might explain the favourable partition coefficients. The two systems having partition coefficients <0.01 were used for further experiments.

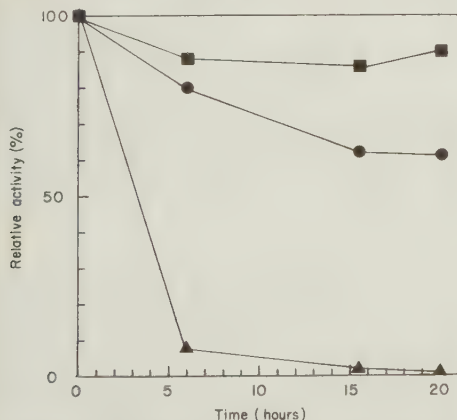
BP, 6-APA and phenylacetic acid partitioned as follows in the 8.9% (w/w) PEG 20000/7.6% (w/w) potassium phosphate system at pH 7.8: BP,  $K=8.3$ ; 6-APA,  $K=1.35$ ; phenylacetic acid,  $K=1.7$ .

### Stability of penicillin acylase in aqueous two-phase systems

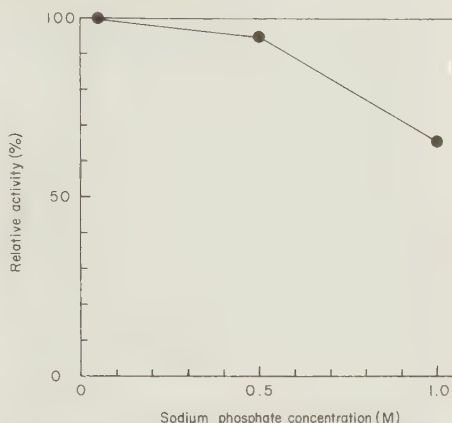
We compared the stability of penicillin acylase in PEG-magnesium sulphate and the PEG-potassium phosphate phase system with  $K < 0.01$  (Figure 1). The stability of the enzyme was very poor in the PEG-magnesium sulphate phase system. In contrast, the PEG-potassium phosphate phase system seemed to confer stability to the enzyme compared with the buffer solution. The PEG-potassium phosphate phase system was therefore chosen for further investigations.

### Effect of phosphate and pH

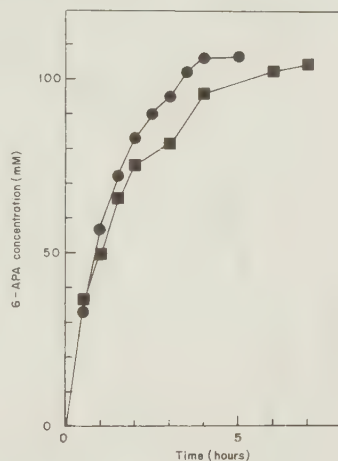
Phosphate destabilized, or inhibited, the activity of penicillin acylase with increasing concentrations so that only 65% of the activity at zero phosphate concentration remained in 1 M phosphate (Figure 2). In the phase system chosen the bottom phase with the enzyme contained



**Figure 1** Stability of penicillin acylase. Aqueous two-phase system: ■, 8.9% (w/w) PEG 20000/7.6% (w/w) potassium phosphate; ▲, 12% (w/w) PEG 3350/10% (w/w) magnesium sulphate; ●, 0.05 M sodium phosphate buffer. The pH was 7.8 and the temperature 37°C. 100% relative activity is equal to 0.79, 0.66 and 0.64 U ml<sup>-1</sup>, respectively



**Figure 2** Relative activity of penicillin acylase in phosphate buffer solutions of increasing strength at pH 7.8 and 37°C. 100% relative activity is equal to 2.2 U ml<sup>-1</sup>



**Figure 3** Conversion of 243 mM benzylpenicillin (BP) to 6-aminopenicillanic acid (6-APA) (measured in samples from total phase system and buffer solution) with penicillin acylase at 37°C and starting pH 8.1. Aqueous two-phase system: ●, 8.9% (w/w) PEG 20000/7.6% (w/w) potassium phosphate; ■, 0.5 M potassium phosphate buffer. The enzyme concentration was 0.3 mg protein ml reaction solution<sup>-1</sup> (10.4 U mg protein<sup>-1</sup>)

~12% (w/w) 0.8 M phosphate, which implies that only 75% of the originally added enzyme activity will be used in such a phase system.

It has been suggested<sup>20</sup> that phosphate might be an intrinsic inhibitor of penicillin acylase at pH 6 whereas 0.1 M phosphate buffer enhances the activity of the enzyme at pH 8. Furthermore, the reaction catalysed by penicillin acylase is an equilibrium one which is shifted towards the formation of BP when the pH falls below 6.<sup>21</sup> Therefore the combined effect of low pH and high phosphate makes it necessary to buffer the system so that the pH does not fall below 7.

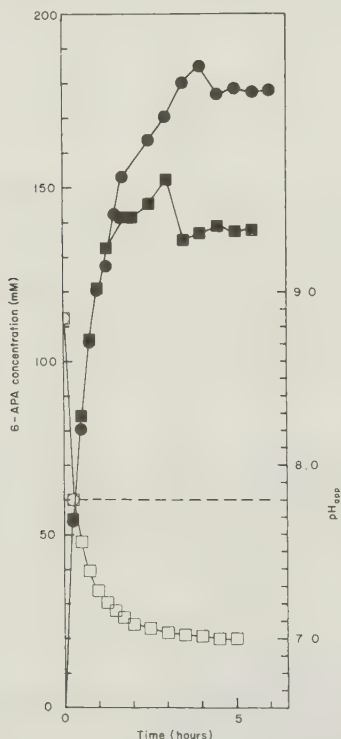
The phase system chosen, containing PEG 20000 and potassium phosphate, should have a built-in buffering capacity obtained from the use of potassium phosphate as a phase component, which means that such a system should need less titration.

### Conversion in buffer versus phase system

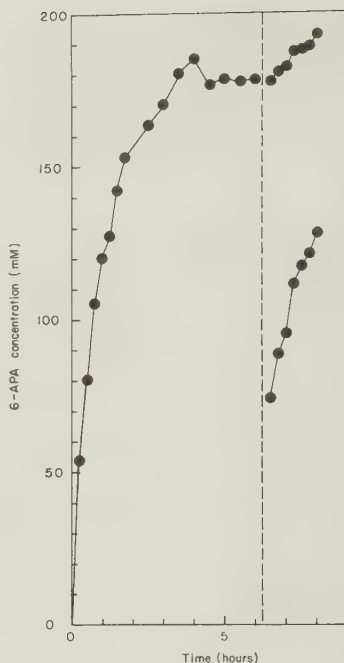
The conversion of BP to 6-APA by penicillin acylase in the selected PEG-potassium phosphate system (holding  $\sim 0.5$  M phosphate) was compared with the conversion in a 0.5 M potassium phosphate buffer (Figure 3). The buffer was chosen to give an equal pH decrease for the conversion as possible. The results indicate that the reaction follows the same kinetic pattern in the phase system as in the buffered solution. In this study a crude preparation of PEG was used. It has been found that the PEG contains inhibitory impurities. However, the data in Figure 3 suggest that penicillin acylase is not sensitive to these impurities.

### Effect of pH

During the course of the reaction (Figure 3) the pH fell to 7.0, in both the buffer and the phase system, in



**Figure 4** Conversion of 243 mM BP to 6-APA (measured in samples from total phase system) at 37°C with and without pH control. 6-APA concentration: ●, with pH control; ■, without pH control. Apparent pH: ○, with pH control; □, without pH control. pH was controlled with 7.5 M NaOH. The enzyme concentration was 1.2 mg protein ml phase system<sup>-1</sup> (10.4 U mg protein<sup>-1</sup>)



**Figure 5** Inhibition of penicillin acylase by products. Conversion of 243 mM BP to 6-APA (measured in samples from total phase system) with penicillin acylase (1.2 mg protein ml phase system<sup>-1</sup>, 10.4 U mg protein<sup>-1</sup>) at 37°C. Top phase composition: 17% (w/w) PEG 20000/4.5% (w/w) potassium phosphate. 6-APA concentration (●) is indicated. BP (3 g) was added to each phase system (25 ml) after it had been divided into two equal portions, as described in the text

which the production of 6-APA is no longer optimal. The effect of pH was investigated in two parallel conversions: in the first there was no pH control and in the second the pH, after falling initially, was titrated to a constant level at 7.8 (Figure 4). In the absence of pH control 6-APA production stopped at a 40 mM (22%) lower level than when the pH was controlled. Clearly, then, it is desirable that the pH be kept at or above 7.8 by titration. However, since the phase system is sensitive to changes by dilution, the titration must be performed with a strong alkaline solution.

### Inhibition of penicillin acylase

The even distribution of 6-APA and phenylacetic acid in the phase system might cause product inhibition of the enzyme.<sup>16</sup> To investigate the effect of product inhibition the following experiment was performed. After the batch conversion, the phase system was divided into two equal portions (Figure 5). To each portion an equal amount of fresh BP was added to ensure that the substrate was available in excess. One sample was given no further treatment and the conversion remained slow, 8.3  $\mu\text{mol min}^{-1}$ . In the second sample the phases were allowed to separate, the top phase was then removed and replaced with fresh top phase, followed by fresh BP addition.



To be able to change the top phase the composition of the top phase was calculated to be: PEG 20000, 17% (w/w), potassium phosphate, 4.5% (w/w). The polymer and the salt were dissolved in water. The procedure of changing the top phase reduced the 6-APA concentration to about half in the phase system, as estimated from the partition coefficient. The conversion was markedly faster,  $36.1 \mu\text{mol min}^{-1}$ , than when the top phase was not changed.

These results confirm that product inhibition is a major factor limiting the conversion of BP to 6-APA.<sup>16</sup>

### Effect of enzyme concentration

Production of 6-APA levelled off at about 105 mM, with a yield of about 40% (Figure 3). The enzyme concentration was therefore increased four-fold (Figure 4), which doubled the initial reaction velocity and increased the final 6-APA concentration by a factor of 1.3. The optimum amount of enzyme to be used in relation to conversion rate and enzyme deactivation remains to be established and is outside the scope of this investigation. However, higher enzyme concentrations than those reported here can be used in aqueous two-phase systems.

Thus, compared to the situation where immobilized enzymes are used in a tank reactor, in which the space occupied by the matrix material determines the enzyme concentration, aqueous two-phase systems allow greater freedom when selecting process variables. In the experiments described below the higher enzyme concentration was used.

### Repeated batch conversions

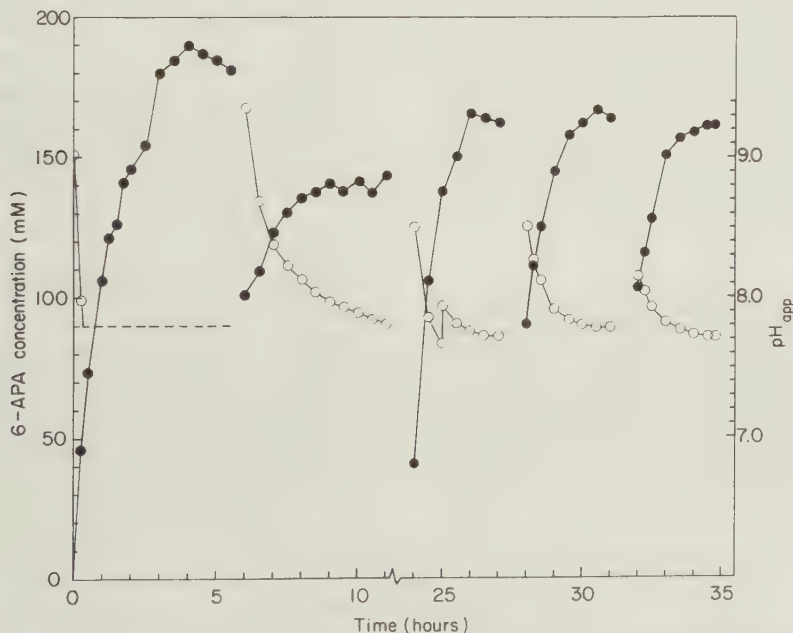
Repeated batch conversions were performed in the PEG-potassium phosphate two-phase system (Figure 6). When 6-APA formation declined the phase system was allowed to separate, the top phase was removed and replaced with new top phase having the composition detailed above and containing fresh substrate. The procedure of changing the top phase, thus supplying the enzyme with fresh substrate and removing the products, was performed four times.

The substrate, BP, was added to the phase system as a dry powder calculated as 10% (w/v). This was equivalent to a concentration of 230 mM based on the total phase volume for the first conversion and on the changed volume for the subsequent conversions. This led to a small increase ( $\leq 4\%$ ) in the volume.

In order to prevent the pH from falling below 7.8 the system was titrated with 7.5 M NaOH. The high concentration was chosen, as mentioned above, to prevent dilution of the phase system.

It has been reported that immobilized penicillin acylase is sensitive to denaturation by strong alkali.<sup>3</sup> During the first conversion, and between the first and second conversions, 7.5 M NaOH was used. For the first two conversions during one hour from the 103 mM level, the calculated specific productivity decreased from  $0.57$  to  $0.31 \mu\text{mol mg protein}^{-1} \text{min}^{-1}$ , and the final 6-APA concentration decreased by 25%.

The yield of 6-APA which was obtained from the top phase when this phase was changed was 34 and 70% for the



**Figure 6** Repeated batch conversion of BP, 10% (w/v), to 6-APA (measured in samples of total phase system) with penicillin acylase ( $1.2 \text{ mg protein ml phase system}^{-1}$ ,  $10.4 \text{ U mg protein}^{-1}$ ) at  $37^\circ \text{C}$ . The BP addition was calculated on the total phase volume for the first time conversion and on the changed volume for the following conversions. After 24 h penicillin acylase was added to re-establish the original starting activity. The added top phase had the composition given in Figure 5. 6-APA concentration (●) and apparent pH (○) are indicated

two first conversions. The low yield of the first conversion depends on the fact that BP was added in relation to the total volume whereas in the second conversion it was added in relation to the changed volume.

To start the third reaction, fresh enzyme was added to re-establish the original activity in the phase system. To overcome the problem of using strong alkaline solution, aqueous two-phase systems offer another possibility. After separation of the phases the titration can be performed in the top phase alone. This procedure protects the enzyme and gives a more gentle alkaline pulse. The procedure was tested between the three last conversions and also during the third conversion. This titration method gave a better result than the method used previously.

The specific productivity measured during one hour from the 103 mM level was found to be almost identical for the last three conversions, i.e. 0.67, 0.64 and 0.65  $\mu\text{mol mg protein}^{-1} \text{min}^{-1}$ , respectively. The yields of 6-APA from the top phase were 47, 52 and 71%, respectively. The increase is partly due to the fact that less of the phase system was changed between each conversion, which reduces the BP addition. However, the final 6-APA level in all conversions was equal at  $\sim 170 \text{ mM}$ .

The specific productivity calculated from the first hour of conversion in reactions one and three was about 1.47 and 1.33  $\mu\text{mol mg protein}^{-1} \text{min}^{-1}$ , respectively. These values should be compared to the results obtained for immobilized penicillin acylase, which is in the range 0.51–4.77  $\mu\text{mol mg protein}^{-1} \text{min}^{-1}$  under the assay conditions.<sup>20</sup>

Figure 6 shows that the starting concentration of 6-APA in the three last conversions increases. This is because the volume ratio in the phase system changes. The bottom phase volume increased because the exact composition of the changed top phase could not be determined at this stage of the investigation. The composition of aqueous two-phase systems containing potassium phosphate changes with temperature as well as with pH because  $\text{HPO}_4^{2-}$  ion is a stronger phase-forming component than  $\text{H}_2\text{PO}_4^-$  ion.<sup>22</sup> The composition and volume ratio of the system may also be influenced by enzyme, substrate and products when used in high concentrations. These effects must also be taken into account when scaling up a process based on aqueous two-phase systems.

## Conclusion

This study has demonstrated that aqueous two-phase systems can be used to recirculate the enzyme penicillin acylase in repeated batch conversions with a productivity in the same range as when immobilized enzyme is used. Aqueous two-phase systems can also be operated continuously in the same way as reactors with immobilized enzymes. The bottom phase containing the enzyme will constitute the stationary phase and the top phase the mobile phase from which the product can be recovered.<sup>13</sup>

Phase systems might have an advantage over immobilized systems in that the enzyme concentration can be optimized

without considering reactor size and matrix volume. Furthermore, inactivated enzyme can be replaced at any time during operation.

The present investigation has, however, also shown the difficulty in finding an optimum phase system that gives a one-sided partition of the enzyme under operational conditions. Further studies are needed to find phase systems or enzymes which give a one-sided partition of the enzyme and are also stable under operational conditions involving pH changes and the use of high concentrations of reactants.

## Acknowledgement

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## References

- Warburton, D., Balasingham, K., Dunnill, P. and Lilly, M. D. *Biochim. Biophys. Acta* 1972, **284**, 278–284
- Lagerlöf, E., Nathorst-Westfelt, L., Ekström, B. and Sjöberg, B. *Methods Enzymol.* 1972, **44**, 759–768
- Carleysmith, S. W. and Lilly, M. D. *Biotechnol. Bioeng.* 1979, **21**, 1057–1073
- Park, J. M., Choi, C. Y., Seong, B. L. and Han, M. H. *Biotechnol. Bioeng.* 1982, **24**, 1623–1637
- Albertsson, P.-Å. *Partition of Cell Particles and Macromolecules* 2nd ed., Wiley-Interscience, New York, 1971
- Bejerinck, M. H. *Zentralblat. Bakteriologie* 1896, **2**, 627, 698
- Kroner, K. H., Hustedt, H. and Kula, M.-R. *Biotechnol. Bioeng.* 1982, **24**, 1015–1045
- Kula, M.-R., Kroner, K. H. and Hustedt, H. *Adv. Biochem. Eng.* 1982, **24**, 73–118
- Mattiasson, B. and Ling, T. G. I. *J. Immunol. Methods* 1980, **38**, 217–223
- Yamazaki, Y. and Suzuki, H. *Rep. Ferment. Res. Inst. Jpn* 1979, **52**, 33–40
- Smeds, A.-L., Veide, A. and Enfors, S.-O. *Enzyme Microb. Technol.* 1983, **5**, 33–36
- Puziss, M. and Hedén, C.-G. *Biotechnol. Bioeng.* 1965, **7**, 355–366
- Hahn-Hägerdal, B., Mattiasson, B., Andersson, E. and Albertsson, P.-Å. *J. Chem. Technol. Biotechnol.* 1982, **32**, 157–161
- Mattiasson, B. and Hahn-Hägerdal, B. in *Immobilized Cells and Organelles* (Mattiasson, B., ed.). CRC Press, Boca Raton, Florida, 1983, vol. 1, pp. 121–134
- Mattiasson, B., Souminen, M., Andersson, E., Häggström, L., Albertsson, P.-Å. and Hahn-Hägerdal, B. in *Enzyme Engineering* (Chibata, I., Fukui, S. and Wingard, L. B., Jr, eds). Plenum Publishing Corporation, New York, 1982, vol. 6, pp. 153–156
- Cole, M. *Biochem. J.* 1969, **115**, 747–756
- Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M. D. *Biochim. Biophys. Acta* 1972, **276**, 250–256
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 1951, **193**, 265–275
- Johansson, G. *Mol. Cell. Biochem.* 1974, **4**, 169–180
- Carleysmith, S. W., Dunnill, P. and Lilly, M. D. *Biotechnol. Bioeng.* 1980, **22**, 735–756
- Svedas, V. K., Margolin, A. L., Borisov, I. L. and Berezin, I. V. *Enzyme Microb. Technol.* 1980, **2**, 313–317
- Albertsson, P.-Å. personal communication



ENZYME ACTION IN POLYMER AND SALT SOLUTIONS:

1. STABILITY OF PENICILLIN ACYLASE IN PEG AND POTASSIUM PHOSPHATE SOLUTIONS IN RELATION TO WATER ACTIVITY

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**SUMMARY**

The stability of penicillin acylase (EC 3.5.1.11) was studied in poly(ethylene glycol) and potassium phosphate solutions. The half-life of the enzymatic activity and the transition temperature determined by differential scanning calorimetry were measured. There was correlation between these two methods for measuring enzyme stability. The enzyme stability could not be related to the water activity, as a measure of solute-solvent interaction. It seems more to be related to the concentration of the solutes and to a minor extent to the molecular weight of PEG. The stabilizing effect of PEG is also discussed in terms of PEG-protein interactions.

**INTRODUCTION**

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) from Escherichia coli catalyses the hydrolysis of natural penicillins and the synthesis of semisynthetic  $\beta$ -lactams. The reaction is a pH-dependent equilibrium which is shifted towards synthesis as pH decreases.

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**Abbreviations:** PEG, poly(ethylene glycol); PA, penicillin acylase; 6-APA, 6-aminopenicillanic acid; BP, benzylpenicillin; DSC, differential scanning calorimetry;  $T_m$ , transition temperature;  $\Delta H_{app}$ , apparent transition heat;  $a_w$ , water activity

PA has two main isoelectric points at 6.7-6.8 and 6.3-6.4 and a molecular weight of approximately 70,000 (1). The kinetics of PA have been investigated for the synthetic reaction. Two mechanisms have been proposed (2).

When used technically for the deacylation of natural penicillins, PA is often immobilized to solid matrices to facilitate the recirculation of the enzyme (3-6). Another method to recirculate an enzyme is to enrich the enzyme in one phase of an aqueous two-phase system. This has been described for a phase system composed of 8.9% (w/w) poly-(ethylene glycol) 20M and 7.6% (w/w) potassium phosphate (7), where the enzyme was partitioned to the potassium phosphate rich bottom phase. After completed reaction, the product was removed together with the top phase and more substrate was added with a new top phase. Productivities comparable to those obtained with immobilized PA were achieved (8).

Besides productivity, operational stability and activity are important parameters for the technical and economical feasibility of a process involving the recirculation of an enzyme.

The introduction of high concentrations of a high molecular weight polymer and high salt concentrations may influence both the stability and the activity of the enzyme.

The melting temperature, defined as the mid point of the transition of optical rotatory melting profiles was found to increase for ribonuclease in the presence of phosphate and sulfate ions (9). Similarly, different polymers have been found to have a stabilizing effect on various enzymes including  $\beta$ -galactose dehydrogenase, acid phosphatase,  $\beta$ -glucosidase (10-12), penicillin acylase (13), and invertase (14).

The stabilization of the protein structure is often interpreted in terms of solvent-solute interactions which prevent the unfolding of the protein molecule (15). Therefore, in the present study, the effects of PEG and phosphate on the stability of penicillin acylase were investigated in relation to the water activity of the solutions. The influence of the

solutes on the activity of the enzyme is reported in a forthcoming communication.

## **MATERIALS AND METHODS**

### Materials

Penicillin acylase (EC 3.5.1.11) (111 U/mL, assay described below) was a generous gift from Fermenta AB, Strängnäs, Sweden. The solution contained 22.75 mg protein/mL solution determined with the Folin method of Lowry et al. (16) using bovine serum albumin (Fraction V, Sigma Chemical Co, St Louis, Mo, USA) as standard. The potassium salt of benzylpenicillin, 6-aminopenicillanic acid and p-dimethylaminobenzaldehyde were obtained from Sigma Chemical Co, St Louis, Mo, USA.

PEG 600 (Mw 570-630) was purchased from Merck, Hohenbrunn, FRG, PEG 1550 (Mw 1300-1600) from Serva, Heidelberg, FRG, PEG 3350 (Mw 3000-3700), PEG 8000 (Mw 7000-9000) and PEG 20M (Mw 16000-24000) from Union Carbide, Danbury, CT, USA. All other chemicals were reagent grade and were obtained from commercial sources.

### Assay of enzymatic activity

All experiments were carried out in a stirred glass vessel (100 mL total volume) thermostated to  $37 \pm 0.1^\circ\text{C}$  with a circulating water bath (Haake, Karlsruhe, FRG) using 25 mL substrate solution. The substrate solution contained 20 mM BP and 100 mM potassium phosphate, pH 7.8. The reaction was started by adding 200  $\mu\text{L}$  of the PA solution after having thermostated the substrate solution for at least 5 minutes. Samples of 0.5 mL were removed and added to 3.5 mL of the analysis solution after 15, 30, 45, 60, 75, 90, 120, 150 and 180 seconds of reaction time.

### Analysis

6-APA was analyzed by the method described by Balasingham et al. (17), where p-dimethylaminobenzaldehyde reacts with 6-APA to form a coloured Schiff's base which can be measured at 415 nm. A Perkin-Elmer Lambda 1 spectrophotometer was used.

### Half-life of enzymatic activity

The half-life of enzymatic activity was measured in PEG and potassium phosphate solutions, using 4.55 mg protein/mL and in the absence of substrate. The solutions also contained 0.3 mg/mL sodium azide to prevent bacterial growth. The temperature was chosen to be 43°C. At this temperature the deactivation of the enzyme was faster, which gave more accurate measurements than at 37°C. Samples were taken from these solutions and assayed for enzymatic activity. The inactivation of PA was assumed to be a first order reaction and the experimental results were fitted by linear regression from which the half-life of the enzymatic activity was estimated.

### DSC analysis

In order to achieve high enough protein concentration for the DSC scans, the enzyme solution was lyophilized, which gave 21.1 mg dry weight per mL enzyme solution lyophilized. The samples for the DSC experiments were prepared by adding the lyophilized enzyme preparation to the polymer and salt solutions to a concentration of 5% (w/w). The samples were then sonicated in a MSE Soniprep 150 Ultrasonic Desintegrator (MSE, Crawly, England) for 2 x 20 seconds at 0°C to achieve a homogeneous dispersion of the protein preparation.

Thermograms were obtained using a Perkin-Elmer DSC-2 calorimeter. The measurements were carried out as previously described (18) using coated aluminium sample containers (Du Pont, Wilmington, De, USA). Before use, the containers were rinsed several times in ethanol and water to remove surplus coating material, which would otherwise contaminate the sample holder of the calorimeter. The containers were dried in an oven at 120°C.

The containers were filled with 10-15 uL samples and sealed by coldwelding in a Du Pont press immediately before the thermograms were run. The containers were weighed before and after the runs to ensure that no leakage had occurred. A Mettler ME22 + BE22 balance (Mettler Instrumente AG, Greifensee-Zürich, Switzerland) with an accuracy of 0.01 mg was used.

The protein content in the sample pan was calculated from the concentration of the lyophilized enzyme preparation in the sample solutions and the sample weight in each sample pan. The dry enzyme content was then multiplied by a factor of 1.08 to obtain the protein content in the pans. This was done in order to compensate for the difference between the protein content in the lyophilized sample and the dry weight obtained per mL enzyme solution after lyophilization.

As reference, a sample container with a piece of silver of about the same heat capacity as the sample was used. The scanning range was 10-100°C, and the heating rate was 10°C/min. The sensitivity was the highest obtainable for the instrument, 0.1 mcal/second, in all experiments.

The transition enthalpy was determined by weighing the peaks in triplets and comparing to a paper area corresponding to 2.51 mJ.

#### Determination of water activity

##### **a) Measurement**

$a_w$  measurements were performed with a Hygroskop DT and a WA-14TH measuring cell, both from Rotronic AG, Zürich, Switzerland. The measuring cell was thermostated to 25°C. The equipment was calibrated with saturated salt solutions of potassium sulfate (0.973), potassium nitrate (0.936), potassium chloride (0.843) and potassium acetate (0.225) (19).

##### **b) Calculations**

$a_w$  in PEG solutions can be calculated using the equation derived by Norrish (20) to predict  $a_w$  in nonelectrolyte solutions:

$$a_w = X_1 \cdot \exp(-K \cdot X_2^2) \quad (1)$$

$X_1$  and  $X_2$  are the molar fractions of water and PEG, respectively, and  $K$  is a constant, which for PEG solutions can be calculated using an equation experimentally found by Chirife and Ferro Fontan (21):

$$K = 1.6 \cdot (MW / 100)^2 \quad (2)$$

When the osmolality of a solute is known, the relationship between  $a_w$  and the osmolality ( $O$ ) can be formulated with two different expressions:

$$a_w = \exp ( O / 55.51 ) \quad (3)$$

This equation is based on the molal freezing point depression ( $K_f = R \cdot T^2 / \Delta H$ ) being constant, and  $T^2 = T \cdot T^*$ , where  $T$  and  $T^*$  are the freezing points of water and the water solution of the solute, respectively.

$$a_w = 55.51 / ( O + 55.51 ) \quad (4)$$

This second equation assumes that the osmolality is equal to  $n_2$  in the general expression for water activity ( $a_w = n_1 / (n_1 + n_2)$ ), where  $n_1$  and  $n_2$  are the number of moles of water and the solute, respectively.

Thus, both equations (3) and (4) give approximate values related to the assumptions made in the formulation of expressions. However, for practical purposes they give useful values at high water activities. Osmolality is a colligative quantity and it is thus possible to calculate  $a_w$  for a polymer solution containing buffering salts by adding several osmolalities. Osmolality data are tabulated for common salts (22).



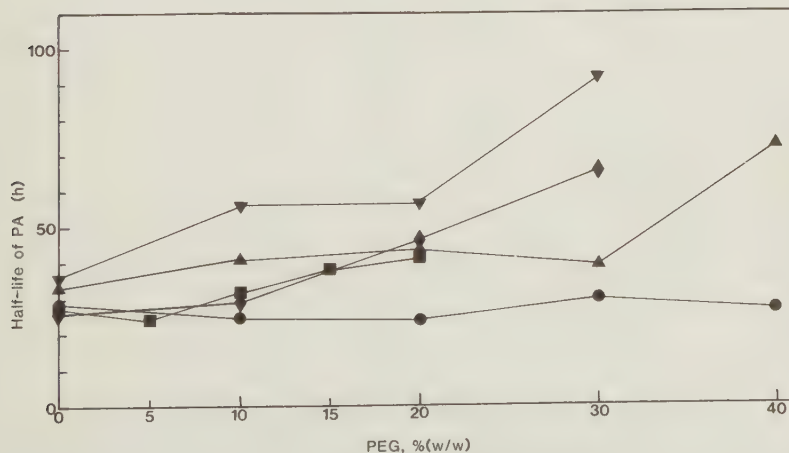
## RESULTS

### Half-life of enzymatic activity in PEG solutions

The stability of PA was studied in PEG solutions with different molecular weight and concentrations of PEG (Figure 1). PEG 600 did not increase the half-life of PA, compared to the reference 0.1 M potassium phosphate buffer, although the concentration was increased to 40 % (w/w). When the molecular weight of PEG was increased, the half-life also increased. A 30% (w/w) solution of PEG 8000 thus increased the half-life of PA by a factor of 3. PEG 20M could only be used up to 20% (w/w) because of viscosity limitations. At this concentration the half-life of PA was lower than in a solution holding the same concentration of PEG 8000.

### Half-life of enzymatic activity in phosphate solutions

The half-life of PA increased by a factor of 4 when the concentration of potassium phosphate was increased from 0.1 to 1.0 M (Figure 2). This increase in stability is larger than what could be obtained with PEG, even at high concentrations.



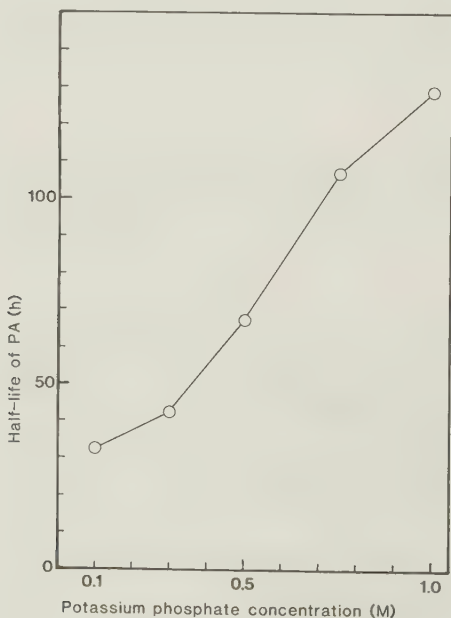
**Figure 1.** Half-life of PA in PEG solutions at 43°C. PEG 600 (—●—), PEG 1550 (—▲—), PEG 3350 (—◆—), PEG 8000 (—▼—) and PEG 20M (—■—). All solutions contained 0.1 M potassium phosphate, pH 7.8.

### DSC measurements

The thermogram for the reference, PA in 0.1M potassium phosphate buffer, shows two peaks with transition temperatures at 66 and 84 °C (Figure 3).

To find out which of the two peaks relates to PA, an enzyme solution was heated with approximately the same rate as in the calorimeter (10°C/min) to 70°C and was then immediately cooled to 0°C. This solution had no remaining activity (experimental data not shown), which indicates that under these conditions PA undergoes an irreversible thermal transition at a temperature below 70°C.

When the PEG 8000 concentration is increased, both transition peaks get broader and the transition temperatures ( $T_m$ ), taken as the peak maximum, increase.



**Figure 2.** Half-life of PA in potassium phosphate solutions at 43°C, pH 7.8.

When the phosphate concentration is increased, no peak broadening can be observed (Figure 4). There is a slight increase in  $T_m$  for peak I, whereas  $T_m$  for peak II first decreases and then increases back to the same  $T_m$  as in the reference.

In the presence of increasing concentrations of phosphate, however, a third peak appears: first, as a shoulder at 0.5 M, then, at 0.75 M and 1 M phosphate,  $T_m$  increases further and becomes a distinct peak.

Thermograms were obtained in duplicate and triplicate for all concentrations and molecular weights of PEG as well as for phosphate. The transition temperatures and apparent transition heats were measured and calculated. The data are summarized as a function of polymer and phosphate concentration. (Figure 5a-d and 6a-d).



**Figure 3.** Thermograms for PEG 8000 solutions. a) Reference solution, 0.1 M potassium phosphate, b) 10%(w/w), c) 20%(w/w) and d) 30%(w/w). All solutions contained 0.1 M potassium phosphate, pH 7.8. The dashed line shows the baseline for determination of the peak area.

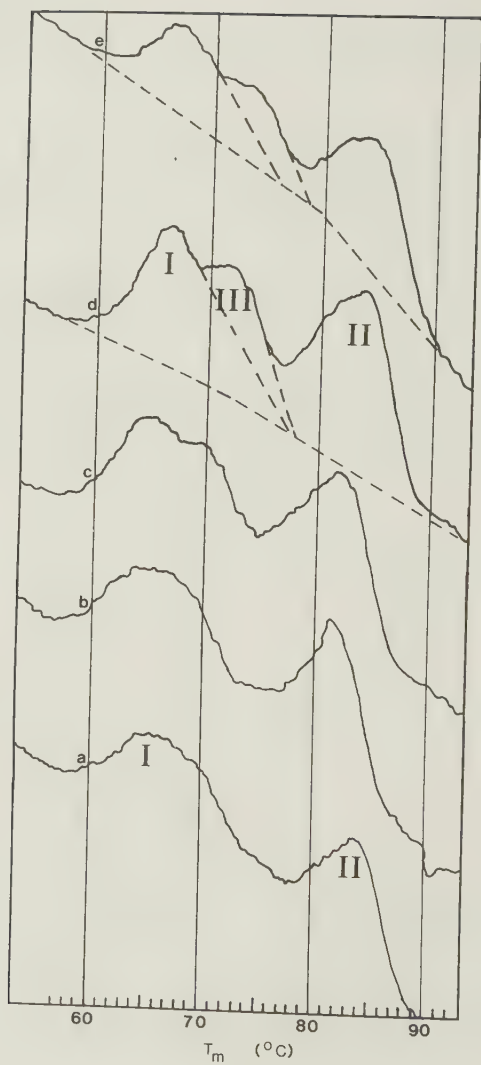
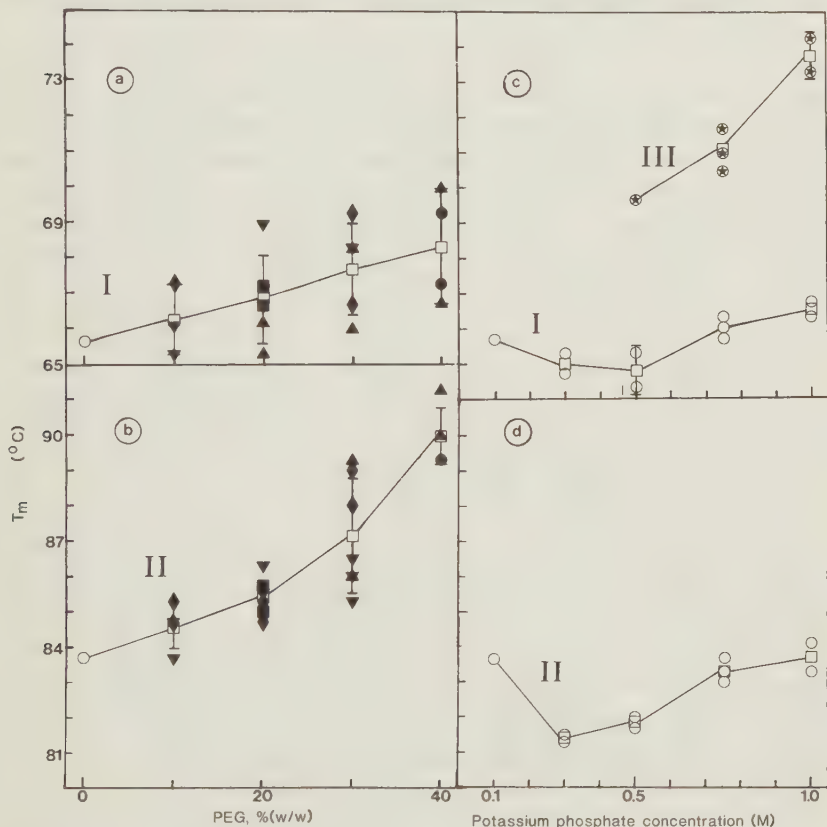


Figure 4. Thermograms for potassium phosphate solutions. a) 0.1 M, b) 0.3 M, c) 0.5 M, d) 0.75 M and e) 1.0 M. The dashed lines indicate the baselines for determination of the peak area.

Despite the scatter of data in the PEG solutions, there is a clear tendency showing that increasing concentrations of polymer increase  $T_m$  (Figure 5 a,b). From the average  $T_m$  the increase is  $2^\circ\text{C}$  for peak I and  $6^\circ\text{C}$  for peak II. The results seem to indicate that the increase in  $T_m$  is related to the concentration of polymer rather than to the molecular weight of the polymer. In the presence of phosphate the situation is quite different (Figure 5 c,d). As indicated in Figure 4,  $T_m$

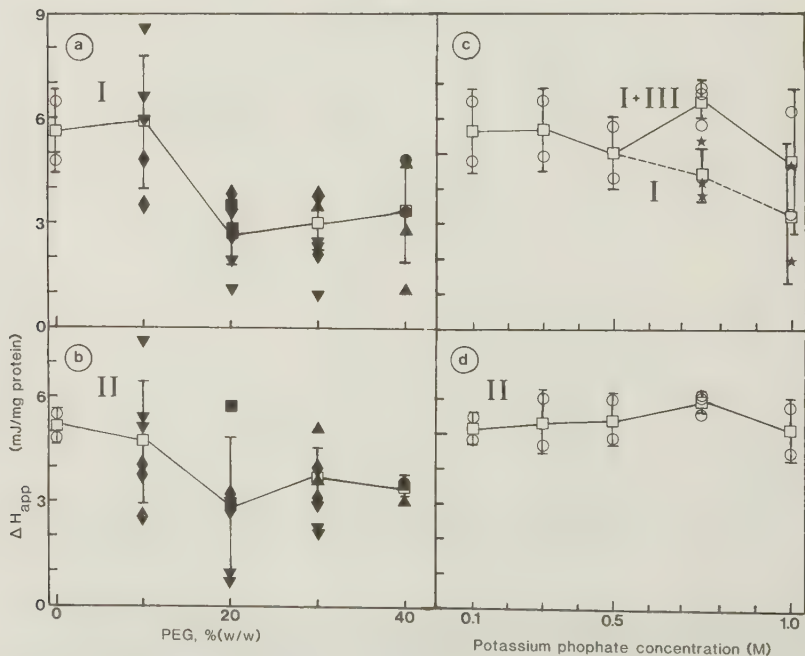


**Figure 5.** Transition temperature ( $T_m$ ) as a function of the PEG (a,b) and potassium phosphate concentrations (c,d). Symbols as in Figure 1 and 2. Peaks by Roman numerals as in Figure 4 and 5. (—□—) indicate average values and bars show standard deviation.

for both peaks first decrease and then increase with increasing phosphate concentration. At the highest phosphate concentration,  $T_m$  is again the same as in the reference.

Peak I, however, which most likely relates to the PA activity, divides into two peaks at 0.5 M phosphate. As the phosphate concentration increases,  $T_m$  for peak III increases  $4^\circ\text{C}$  from  $69.5$  to  $73.5^\circ\text{C}$ . As the thermal transition resulting in peak III has been covered in peak I, the real increase in  $T_m$  is  $8^\circ$  from  $65.5$  to  $73.5^\circ\text{C}$ .

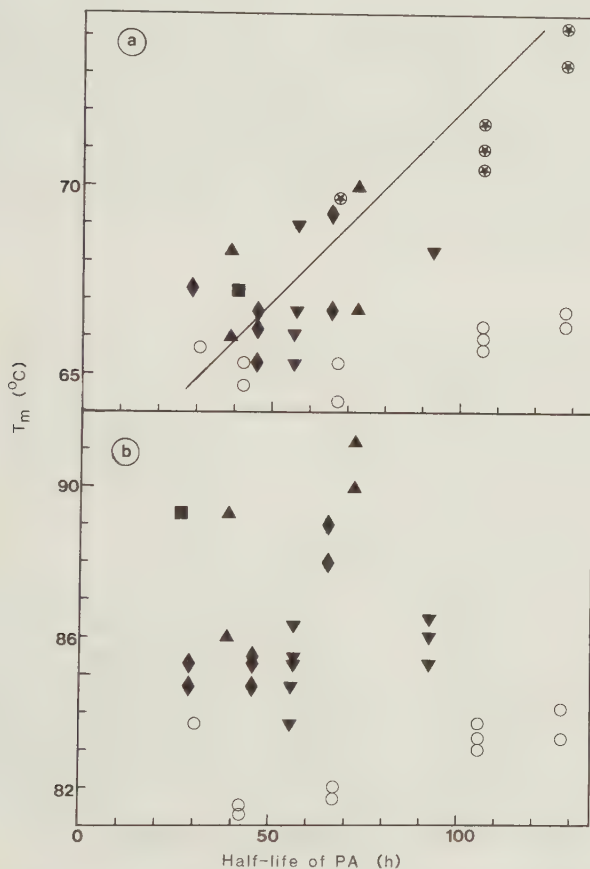
$\Delta H_{\text{app}}$  has been estimated in relation to the total amount of protein in the samples (Figure 6a-d). There is a slight indication that  $\Delta H_{\text{app}}$  decreases when the concentration of PEG increases. It seems to be more so for peak I than for peak II. In phosphate the  $\Delta H_{\text{app}}$  hardly seems to be affected.



**Figure 6.** Apparent transition heat ( $\Delta H_{\text{app}}$ ) as a function of PEG (a,b) and potassium phosphate concentrations (c,d). Symbols as in Figure 5.



The relation between the increase in  $T_m$  for the protein and the increase in half-life of enzymatic activity at different concentrations of phosphate and PEG was also investigated (Figure 7). For peak II the scatter of data indicates that  $T_m$  has no relation to the half-life of the enzymatic activity.

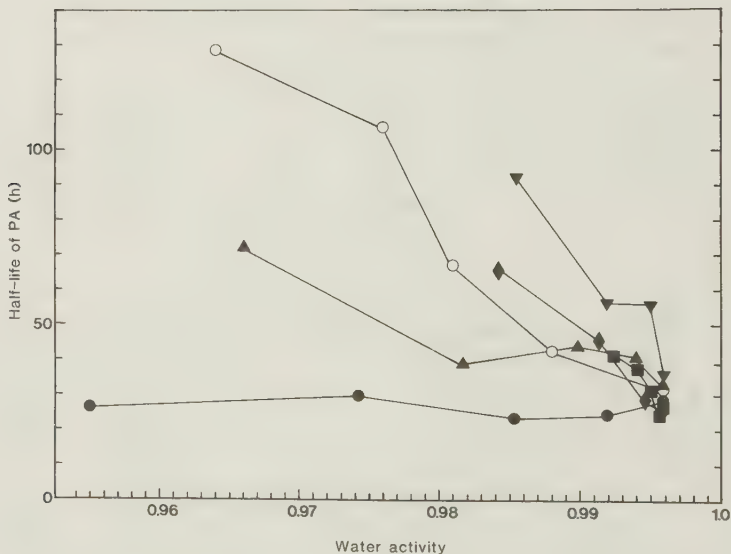


**Figure 7.** Transition temperature ( $T_m$ ) as a function of the half-life of PA for peaks I, III (a) and peak II (b). (⊗) shows peak III. Symbols as in Figure 1 and 2. The line represents the linear regression of values from peak I for the PEG solutions and for the potassium phosphate solutions with a concentration below 0.5 M. The values for peak III were used 0.5 M and above.

When peak I and peak III are considered together - peak III as being part of peak I at low concentrations of solute - there is a correlation between the increase in  $T_m$  and the half-life of the enzymatic activity.

### Water activity

Finally, the relation between the half-life of the enzymatic activity and the water activity of the solute containing solutions was investigated (Figure 8). The water activity data were both calculated and measured (Table I) and the agreement between the differently obtained data is reasonable. The calculated data are used in the further discussion. Only for the phosphate containing solutions there is a relation between decrease in water activity and increase in



**Figure 8.** Half-life of PA in PEG and potassium phosphate solutions related to the water activity. Symbols as in Figures 1 and 2.

half-life of enzymatic activity. This is trivial, however, as increasing concentrations of phosphate decrease the water activity and increase the half-life of the enzymatic activity.

For the PEG containing solutions the situation is different. PEG 600, which decreases the water activity most, hardly influences the half-life of the enzymatic activity. On the other hand, PEG 8000 increases the half-life of the enzymatic activity considerably, despite the fact that it hardly influences the water activity of the solution.

**Table 1.**

Water activity in PEG and potassium phosphate solutions. All the PEG solutions contain 0.1 M potassium phosphate, pH 7.8. The temperature for the calculations and the measurements was 25°C.

|                       | CALCULATED   |              | MEASURED |
|-----------------------|--------------|--------------|----------|
|                       | Equation (1) | Equation (2) |          |
| PEG 600, 40% (w/w)    | 0.955        | 0.955        | 0.947    |
| PEG 1550, 40 % (w/w)  | 0.966        | 0.966        | 0.958    |
| PEG 3350, 30 % (w/w)  | 0.984        | 0.984        | 0.978    |
| PEG 8000, 30 % (w/w)  | 0.985        | 0.986        | 0.979    |
| PEG 20000, 20 % (w/w) | 0.992        | 0.992        | 0.983    |
| K- phosphate, 1.0 M   | 0.963        | 0.964        | 0.975    |
| K-phosphate, 0.5 M    | 0.981        | 0.981        | 0.980    |

## DISCUSSION

The stabilization of enzymes has been given much attention. The thermal stability of proteins and enzymes has been discussed in thermodynamic terms (23). An extensive literature review on the stability of soluble enzymes has been presented (24). The stability of enzymes in relation to their operational stability and especially what can be done to stabilize them against thermal inactivation has also been reviewed (25).

The tertiary structure of proteins is determined by covalent bonds, hydrogen bonds and hydrophobic interactions. Of these, primarily, the hydrogen bonds and hydrophobic interactions are responsible for the stability against unfolding.

When proteins have been stabilized by the addition of solutes, it has been ascribed both to the strengthening of hydrogen bonds (26-27) and to the strengthening of hydrophobic interactions (9,15,28-29). Based on thermodynamic analysis the inhibition of protein unfolding has been suggested to be the result of solute-solvent interactions (15,28). A similar conclusion was arrived at from investigations of diffusion coefficients in solutions of mono-, di- and tri-saccharides (30).

With this background an attempt was made in the present investigation to relate the water activity of a solution to the stability of the protein. A technical preparation of penicillin acylase was investigated, since its performance had previously been studied in an aqueous two-phase system (7).

The stability of the enzyme was measured in two ways: as the half-life of the enzymatic activity and as  $T_m$  measured by DSC.  $T_m$  obtained by DSC is closely related to the temperature of heat denaturation, and is thus a measure of the stability of the enzyme against heat denaturation. Since a technical enzyme preparation was used, the thermograms showed several heat transitions indicating the presence of other macromolecules. There is, however, a good agreement between  $T_m$  of

one of the peaks and the half-life of the enzymatic activity. Thus, the presence of PEG and phosphate stabilizes the enzyme both in terms of prolonged enzymatic activity and in terms of increased resistance against heat denaturation.

Lyophilized enzyme had to be used in the DSC experiments in order to obtain a sufficiently high protein concentration in the samples. The lyophilized enzyme preparation was sonicated in order to obtain a homogeneous dispersion. It is of course questionable to draw conclusions from data on an enzyme preparation that is difficult to dissolve. Nevertheless, there is a linear relation between the transition temperature and the half-life of the enzymatic activity, indicating that these two qualities are both measures of enzyme stability.

The DSC data in the present investigation did not allow any thermodynamic analysis of forces involved in stabilizing the protein structure. The thermal transition did not occur under equilibrium conditions and was irreversible.

When the data were analyzed in terms of decreased water activity, no relationship was found between the stabilizing effect of PEG and a reduced water activity of the solution. The stabilization of PA in the presence of PEG seems rather to be related to molecular weight and concentration of PEG.

The stabilizing effect of PEG could be interpreted in two ways; (i), PEG would interact with the solvent which would stabilize the protein structure against unfolding as has been proposed for sugars (29) and polyols (31); (ii), PEG would interact direct with the protein molecule and stabilize it against unfolding. The first alternative was the basis for studying the relation between the stability of the enzyme and the water activity of the solution in the present investigation. PEG has been found to strongly interact with water (32) and the strong water binding has been proposed to initiate fusion with PEG (33). The results of the present investigation show no relationship between the water binding capacity of PEG, measured as water activity, and its ability to induce protein stability.

The thermograms obtained in the present investigation show that in presence of PEG, the transition peaks become broader as the concentration of the polymer becomes higher. This is an indication of direct interaction between the protein and PEG.

It has also been observed that proteins which have been enriched in a PEG rich top phase of an aqueous two-phase system, behaved differently in a following chromatography purification step as compared to the same protein purified without a phase system (Tony Atkinson, personal communication). This effect has been ascribed to specific interactions between PEG and the protein surface, which are supposed to change the surface properties of the protein.

The fact that PEG 20M stabilizes PA less than PEG 8000 does, might be a further indication that the stability is brought about by interactions between the protein and the polymer. PEG 20M is a copolymer of 2-3 PEG 8000, which might make part of the polymers branched, whereas PEG 8000, and all the other PEG preparations, are linear polymers. A linear PEG can be assumed to interact easier with a protein surface.

The stabilizing effect of phosphate agrees well with what has previously been found for ribonuclease (9), and the data of the present investigation do not allow any further interpretation of the mechanisms for stabilization.

In conclusion, the effects of PEG and phosphate on the stability PA can not be related to a solute-solvent interaction, measured as the water activity. In the case of PEG, the stabilizing effect rather seems to be due to an interaction between the polymer and the protein.

Finally, the results show that when the enzyme is re-circulated in an aqueous two-phase system, its stability can be greatly enhanced.



## ACKNOWLEDGEMENT

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## REFERENCES

1. Kutzbach, C. and Rauenbusch, E. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 45-53
2. Kasche, V., Haufler, U. and Zöllner, R. (1984) Hoppe-Seyler's Z. Physiol. Chem. 365, 1435-1443
3. Warburton, D., Balasingham, K., Dunnill, P. and Lilly, M.D. (1972) Biochim. Biophys. Acta 284, 278-284
4. Lagerlöf, E., Nathorst-Westfelt, L., Ekström, B. and Sjöberg, B. (1976) Methods Enzymol. 44, 759-768
5. Carleysmith, S.W. and Lilly, M.D. (1979) Biotechnol. Bioeng. 21, 1057-1073
6. Park, J.M., Choi, C.Y., Seong, B.L. and Han, M.H. (1982) Biotechnol. Bioeng. 24, 1623-1637
7. Andersson, E., Mattiasson, B. and Hahn-Hägerdal, B. (1984) Enzyme Microb. Technol. 6, 301-306
8. Carleysmith, S.W., Dunnill, P. and Lilly, M.D. (1979) Biotechnol. Bioeng. 22, 735-756
9. von Hippel, P.H. and Wong, K.-Y. (1965) J. Biol. Chem. 240, 3909-3923
10. Gianfreda, L. and Greco, G. Jr. (1981) Biotechnol. Lett. 3, 33-38
11. Greco, G. Jr. and Gianfreda, L. (1981) Biotechnol. Bioeng. 23, 2199-2210
12. Alfani, F., Cantarella, M., Cirielli, G. and Scardi, V. (1984) Biotechnol. Lett. 6, 345-350
13. Greco, G. Jr., Veronese, F., Largajolli, R. and Gianfreda, L. (1983) Eur. J. Appl. Microbiol. Biotechnol. 18, 333-338
14. Monsan, P. and Combes, D. (1984) in Enzyme Engineering 7 (Laskin, A.I., Tsao, G.T. and Wingard, L.B., Jr., eds.), Ann.N.Y.Acad.Sci. 434, 48-60
15. Gekko, K. and Timasheff, S.N. (1981) Biochemistry 20, 4677-4686
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

17. Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M.D. (1972) *Biochim. Biophys. Acta* 276, 250-256
18. Hägerdal, B. and Martens, H. (1976) *J. Food Sci.* 41, 933-937
19. Greenspan, L. (1977) *J. Res. Nat. Bur. Stand. (U.S.)* 81A, 89-96
20. Norrish, R.S. (1966) *J. Food Technol.* 1, 25-39
21. Chirife, J. and Ferro Fontan, C. (1980) *J. Food Sci.* 45, 1717-1719
22. *Handbook of Chemistry and Physics*, CRC Press Inc., Cleveland, USA.
23. Brandt, J.F. (1967) In *Thermobiology* (Rose, A.H., ed.) pp. 25-72, Academic Press, London, New York
24. Schmid, R.D. (1979) in *Advances of Biochemical Engineering* (Fiechter, A., ed.), Vol.12, pp. 41-118, Springer-Verlag, Berlin
25. Klibanov, A.M. (1983) *Adv. Appl. Microbiol.* 29, 1-28
26. Gerlsma, S.Y. (1968) *J. Biol. Chem.* 243, 957-961
27. Gerlsma, S.Y. (1970) *Eur. J. Biochem.* 14, 150-153
28. Back, J.F., Oakenfull, D. and Smith, M.B. (1979) *Biochemistry* 18, 5191-5196
29. Uedaira, H and Uedaira, H. (1980) *Bull. Chem. Soc. Jpn.* 53, 2451-2455
30. Uedaira, H. and Uedaira, H. (1985) *J. Solution Chem.* 14, 27-34
31. Gekko, K. (1982) *J. Biochem.* 91, 1197-1204
32. Tilcock, C.P.S. and Fisher, D. (1982) *Biochim. Biophys. Acta* 688, 645-652
33. Fisher, D. and Goodall, A.H. (1981) *Techniques Cell. Physiol.* Pl15, 1-36

# III

## ENZYME ACTION IN POLYMER AND SALT SOLUTIONS:

### 2. ACTIVITY OF PENICILLIN ACYLASE IN PEG AND POTASSIUM PHOSPHATE SOLUTIONS IN RELATION TO WATER ACTIVITY

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#### SUMMARY

The activity of penicillin acylase (EC 3.5.1.11) was investigated in poly(ethylene glycol) and potassium phosphate solutions. Increasing concentrations of both solutes decreased the enzymatic activity. Low molecular weight PEG reduced the PA activity more than high molecular weight PEG. The decrease in enzymatic activity seemed to relate to the decrease in water activity in solutions containing PEG irrespective of the molecular weight of the polymer. The decrease in enzymatic activity in potassium phosphate solutions was lower than in PEG solutions and did not fit the water activity relation. The decreased enzymatic activity is discussed in relation to the enzyme stability in these solutions.

#### INTRODUCTION

The recirculation of penicillin acylase has been studied in an aqueous two-phase system composed of 8.9% (w/w) poly(ethylene glycol) 20M and 7.6% (w/w) potassium phosphate, where the enzyme is partitioned to the phosphate rich bottom phase (1). Productivities comparable to those obtained when

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**Abbreviations:** PEG, poly(ethylene glycol); PA, penicillin acylase; 6-APA, 6-aminopenicillanic acid; BP, benzylpenicillin;  $a_w$ , water activity

the enzyme is immobilized in solid matrices were achieved (2). Besides productivity, also stability and activity of the enzyme play an important role for the feasibility of a process. The introduction of high concentrations of polymer and salt can be expected to change both these parameters.

The influence of PEG and potassium phosphate on the stability of PA has been reported in a preceeding communication (3). Increasing concentrations of both solutes increase the stability of the enzyme measured as the half-life of the enzymatic activity and transition temperature for thermal denaturation. Furthermore, the stability measured as increase in half-life of enzymatic activity increased with increasing molecular weight of PEG. An attempt was made to relate the improved stability to the decrease in water activity brought about by the solutes. The relation is trivial for potassium phosphate, but for PEG no relation could be established.

The present communication describes the influence of PEG and potassium phosphate on the activity of PA, and its relation to the decrease in water activity caused by the solutes.

## **MATERIAL AND METHODS**

### Materials

Penicillin acylase (EC 3.5.1.11) (111 U/mL, assay described below) was a generous gift from Fermenta AB, Strängnäs, Sweden. The solution contained 22.75 mg protein/mL determined with the Folin method of Lowry *et al.* (4) using bovine serum albumine (Fraction V, Sigma Chemical Co, St Louis, Mo, USA) as standard. The potassium salt of benzylpenicillin, 6-aminopenicillanic acid and p-dimethylaminobenzaldehyde were obtained from Sigma Chemical Co, St Louis, Mo, USA.

PEG 600 (Mw 570-630) was purchased from Merck, Hohenbrunn, FRG, PEG 1550 (Mw 1300-1600) from Serva, Heidelberg, FRG, PEG 3350 (Mw 3000-3700), PEG 8000 (Mw 7000-9000) and PEG 20M (Mw 16000-24000) from Union Carbide, Danbury, CT, USA. All other chemicals were reagent grade and were obtained from commercial sources.

### Assay of enzymatic activity

All experiments were carried out in a stirred glass vessel (100 mL total volume) thermostated to  $37 \pm 0.1^\circ\text{C}$  with a circulating water bath (Haake, Karlsruhe, FRG) using 25 mL substrate solution. The substrate solution contained 20 mM BP and 100 mM potassium phosphate, pH 7.8. The reaction was started by adding 200  $\mu\text{L}$  of the PA solution after having thermostated the substrate solution for at least 5 minutes. Samples of 0.5 mL were removed and added to 3.5 mL of the analysis solution after 15, 30, 45, 60, 75, 90, 120, 150 and 180 seconds of reaction time.

### Analysis

6-APA was analyzed by the method described by Balasingham et al. (5), where p-dimethylaminobenzaldehyde reacts with 6-APA to form a coloured Schiff's base which can be measured at 415 nm. A Perkin-Elmer Lambda 1 spectrophotometer was used.

### Water activity

The decrease in water activity resulting from the presence of PEG and potassium phosphate was both measured and calculated as previously described (3).

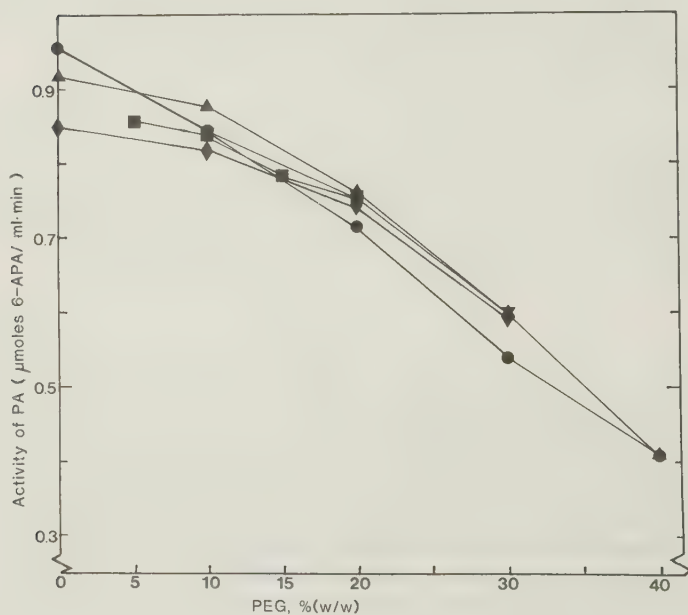
## **RESULTS**

### Activity of PA in PEG solutions

The activity of PA was first studied in a 20% (w/w) solution of PEG 20M, which is the most viscous solution used in this investigation. A series of experiments was performed, where the stirring speed in the reaction vessel was the only parameter which was varied (experimental data not shown). A stirring speed, where the reaction rate was independent of the stirring, was chosen for all further experiments.

The activity of PA was then studied in solutions of PEG holding different concentrations and different molecular weights (Figure 1). There is an obvious relation between the activity of the enzyme and the concentration of PEG; the

higher the concentration, the lower the enzymatic activity. On the other hand, the molecular weight of PEG does not significantly influence the enzymatic activity. There seems to be a tendency showing that a low molecular weight PEG decreases the enzymatic activity more than a high molecular weight PEG does. Thus, a high concentration of a low molecular weight PEG reduces the enzymatic activity the most, whereas a low concentration of a high molecular weight PEG hardly influences the enzymatic activity at all. Only 43% of the PA activity is left in a 40 %(w/w) PEG 600 solution, whereas almost 90 % of the PA activity remains in a 20 %(w/w) PEG 20M solution.



**Figure 1.** Activity of PA in PEG solutions. All solutions contained 0.1 M potassium phosphate buffer. pH 7.8, 37°C. PEG 600 (●—●), PEG 1550 (▲—▲), PEG 3350 (◆—◆), PEG 8000 (▼—▼) and PEG 20M (■—■).

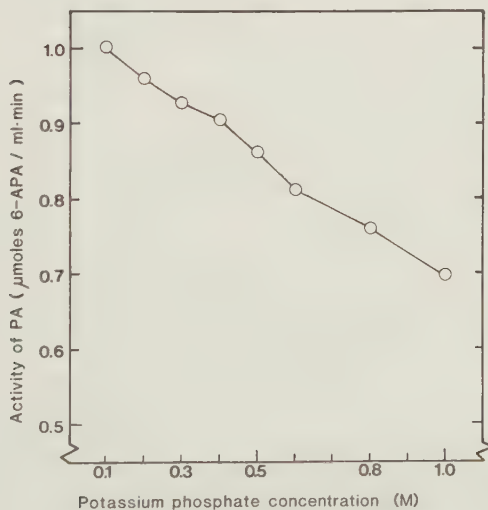


### Activity in potassium phosphate solutions

The influence of increasing concentrations of potassium phosphate on the PA activity was also investigated (Figure 2). The enzymatic activity decreased by more than 30 % when the potassium phosphate concentration was increased from 0.1 to 1.0 M.

### Activity versus stability of PA

The relation between the activity and the stability measured as the half-life of PA activity is shown in Figure 3. Most stability and the least loss of enzymatic activity is gained in the presence of potassium phosphate and PEG 8000. With decreasing molecular weight, the presence of PEG becomes less and less favourable for the relation between the stability and activity. Thus, PEG 600 reduces the activity considerably without any improvement in stability.

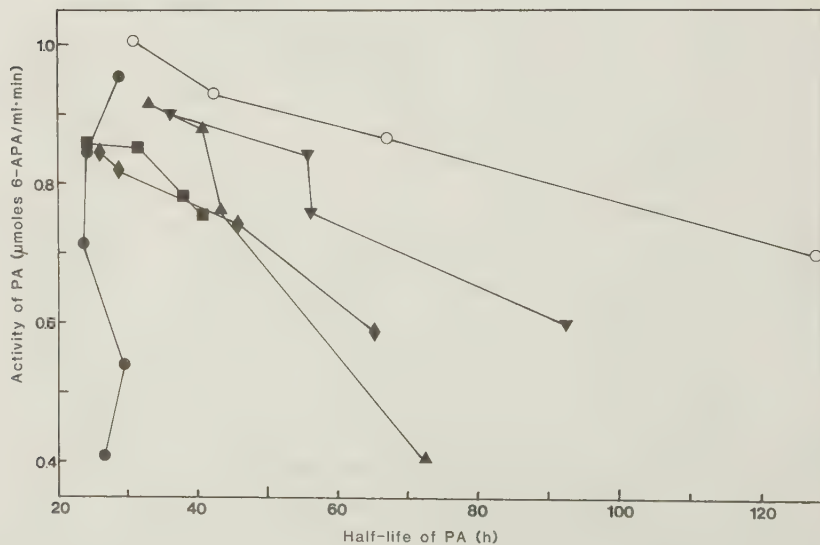


**Figure 2.** Activity of PA in potassium phosphate solutions. pH 7.8, 37°C.

When the stability of the enzyme is measured as the transition temperature for the thermal denaturation, the relation to the decrease in activity becomes somewhat different (Figure 4). Again, the most is gained and the least is lost in the presence of phosphate. There seems, however, to be little difference between the different molecular weight PEG's as opposed to when the half-life of the enzymatic activity is considered.

### Water activity effects

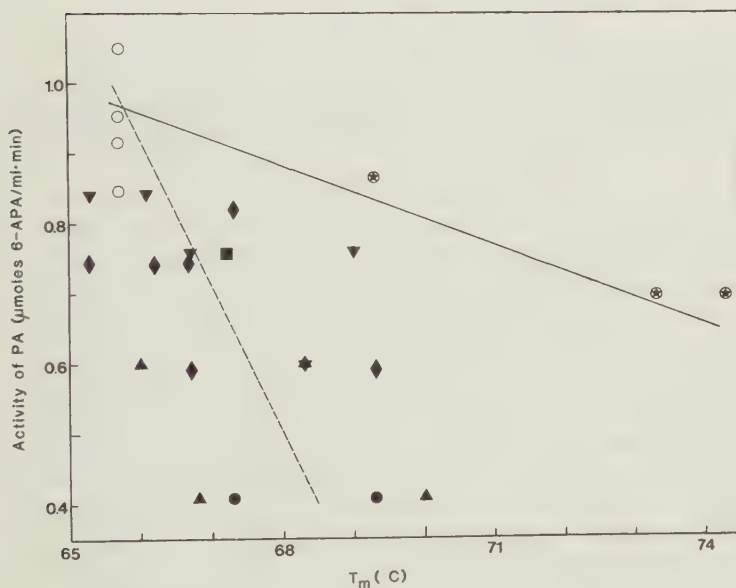
In order to elucidate if the loss in enzymatic activity found at high polymer and high salt concentrations could be related to a common physical property,  $a_w$  for PEG and potassium phosphate solutions was both calculated and measured (3). A reasonable agreement between the calculated and measured data was found and the calculated data are used in further discussions.



**Figure 3.** Activity of PA as a function of half-life of enzymatic activity. Half-life data from reference (3). Symbols as in Figure 1 and 2.

The enzymatic activities in the PEG and the potassium phosphate solutions were plotted against  $a_w$  in the solutions (Figure 5). At the same  $a_w$ , potassium phosphate decreases the enzymatic activity less than PEG does.

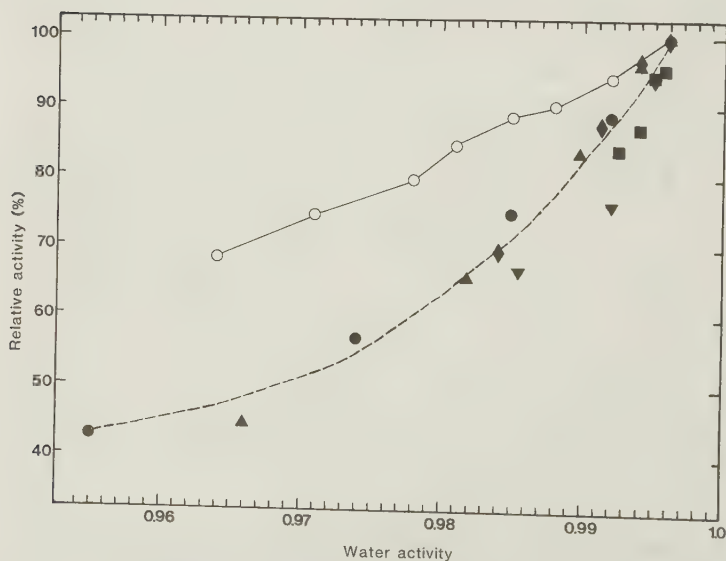
For the PEG solutions, there seems to be a relation between  $a_w$  and the enzymatic activity, since the different symbols representing the different molecular weights of PEG overlap each other and fit one curve. This indicates that a certain  $a_w$  in a PEG solution decreases the enzymatic activity to the same extent, irrespective of the molecular weight of PEG.



**Figure 4.** Activity of PA as a function of transition temperature ( $T_m$ ).  $T_m$  data from reference (3). Symbols as in Figure 1 and 2 except ( $\otimes$ ), which represents peak III occurring at a potassium phosphate concentration of 0.5 M and above. The line (—) is the linear regression for potassium phosphate solutions and the dashed line (---) the linear regression for the PEG solutions.

## DISCUSSION

So far, relatively little has been reported on the relation between enzymatic activity and water activity. Enzymatic reactions have been studied in food materials dried to various water contents (6). Hydrolytic as well as non-hydrolytic enzymes have been studied, and it has been proposed that water, even in rather dry materials, primarily serves as a diffusion medium, bringing the substrate in contact with the enzyme (7).



**Figure 5.** Relative activity of PA in PEG and potassium phosphate solutions as a function of the water activity. 100 % relative activity refers to the activity in 0.1 M potassium phosphate in each experiment. Symbols as in Figures 1 and 2.

When the substrate is non-aqueous and water only, like e.g. in lipolysis, is needed as a reactant, enzymatic reactions can take place at extremely low  $a_w$  which has been found for lipid degradation in full-fat milk powder (8).

For enzyme-catalyzed equilibria involving water as a reactant, it has been shown that hydrolytic enzymes can act synthetically when the water activity is low enough (9-10). When enzymes act in organic solvents with an extremely low water content, their specificity is changed as was demonstrated for porcine pancreatic lipase (11). This was interpreted in terms of a higher rigidity of the protein structure, which was no longer being reactive towards bulky side-chains.

In a solution with water activity of 0.95, which is the lowest water activity studied in this investigation, the water concentration is 52.7 M.

Another way to determine the availability of water has been to measure the amount of freezable water by differential scanning calorimetry (12). Water is supposed to bind both to the terminal hydroxyl moieties and to the ether-linked oxygen atoms in PEG. The amount of free water in a 40% (w/w) PEG 600 solution was estimated to be 28.8% (w/w). The corresponding figures for 40% (w/w) PEG 1500, 30% (w/w) PEG 4000 and 30% (w/w) PEG 6000 were 24.4% (w/w), 40.0% (w/w) and 37.6% (w/w), respectively. Consequently, there should be enough water available for the deacylation reaction, where water acts as a donor of a proton to 6-APA and a hydroxyl group to phenylacetic acid when BP is cleaved. Therefore, the range of water activity studied in the present investigation by additions of PEG and phosphate, falls in the range where water primarily serves as a diffusion medium for the substrate and the enzyme.

High concentrations of high molecular weight PEG increase the viscosity of the reaction medium. For glucoamylase, it was found that an increased viscosity decreased the apparent  $K_m$ , i.e. the initial reaction rate increased (13). This was suggested to be due to an exclusion effect. A similar result was

reported for the enzyme hyaluronate lyase in 5% (w/v) PEG 3350 (14). The  $K_m$  value decreased from 38.5  $\mu\text{g/mL}$  in a buffer solution to 28  $\mu\text{g/mL}$  in the PEG solution.

The results of the present investigation can not be directly compared since kinetic constants were not determined. The activity was determined as the initial reaction rate, which decreased when the PEG concentrations increased.

The viscosity of the top phase of an aqueous two-phase system holding 17% (w/w) PEG 20M has been determined to be 136 mPa·s (Anneli Svensson, unpublished data). In contrast, a 20% (w/w) PEG 600 solution has a viscosity of 3 mPa·s. 20% PEG 20M only decreased the enzymatic activity 10%, whereas 20% PEG 600 decreased the activity 25%. Furthermore, all experiments in this investigation were carried out at a stirring rate where the enzymatic reaction was independent of mixing. Thus, the decreased enzymatic activity, observed at high PEG concentrations in the present study, does not seem to be related to diffusion limitations in the PEG solutions.

The decrease in activity of PA in solutions holding PEG and potassium phosphate has previously been shown to be accompanied by an increased stability, measured as half-life of enzymatic activity and temperature of heat transition, respectively (3). Therefore, the decrease in enzymatic activity might be interpreted in terms of an increased rigidity of the protein molecule. Whether this is brought about by an interaction between the protein molecules and the solutes or by an interaction between the solutes and the solvent can not be discriminated from the present data. There was no relation between the stability of PA and the water activity in PEG solutions, taken as a measure of solute-solvent interaction. Furthermore, previous calorimetry studies (3), as well as protein purification studies including PEG (Tony Atkinson, personal communication), indicate a direct interaction between the enzyme molecules and PEG. On the other hand, the present investigation indicates a relation between reduced water activity in PEG solutions and reduced enzymatic activity irrespective of the molecular weight of PEG.



The fact that the effect of water activity in potassium phosphate solutions falls outside the PEG relations for both enzymatic activity and stability further demonstrates that water activity as a measure of solute-solvent interaction is not the only parameter influencing the enzyme performance.

The present study shows that when the enzyme PA is recirculated in an aqueous two-phase system of 8.9% (w/w) PEG 20M and 7.6% (w/w) potassium phosphate, about 30% of its activity must be expected to be lost due to environmental conditions. At the same time, however, the half-life of the enzymatic activity can be expected to be increased about three times (3).

#### ACKNOWLEDGEMENT

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#### REFERENCES

1. Andersson, E., Mattiasson, B. and Hahn-Hägerdal, B. (1984) *Enzyme Microb. Technol.* 2, 301-306
2. Carleysmith, S.W., Dunnill, P. and Lilly, M.D. (1980) *Biotechnol. Bioeng.* 22, 735-756
3. Andersson, E. and Hahn-Hägerdal, B. (submitted for publication)
4. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275
5. Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M.D. (1972) *Biochim. Biophys. Acta* 276, 250-256
6. Acker, L. and Kaiser, H. (1959) *Z. Lebensmittel-Unters. u. -Forsch.* 110, 349-356
7. Acker, L.W. (1969) *Food Technol.* 23, 27-40
8. Andersson, R.E. (1980) *Appl. Environ. Microbiol.* 39, 36-40
9. Klibanov, A.M., Samokhin, G.P., Martinek, K. and Berezin, I.V. (1977) *Biotechnol. Bioeng.* 19, 1351-1361
10. Halling, P.J. (1984) *Enzyme Microb. Technol.* 6, 513-516

11. Zaks, A. and Klibanov, A.M. (1984) Science 224, 1249-1251
12. Tilcock, C.P.S. and Fisher, D. (1982) Biochim. Biophys. Acta 688, 645-652
13. Hinsch, B. and Kula, M.-R. (1978) Biochim. Biophys. Acta 525, 392-398
14. Laurent, T.C. (1971) Eur. J. Biochem. 21, 498-506

# $\alpha$ -Amylase production in aqueous two-phase systems with *Bacillus subtilis* IV

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The production of  $\alpha$ -amylase (1,4- $\alpha$ -D-glucan glucanohydrolase, EC 3.2.1.1) by *Bacillus subtilis* has been studied in repeated batch fermentations in aqueous two-phase systems. In a phase system composed of PEG 600, 8% (w/w), PEG 3350, 5% (w/w)/Dextran T 500, 2% (w/w), 82% of the enzyme partitioned to the top phase. The enzyme concentration in the top phase reached 0.85–1.35 U ml<sup>-1</sup> during the fermentations compared with 0.58 U ml<sup>-1</sup> in the reference fermentation. In the phase system composed of PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w), 73% of the enzyme partitioned to the top phase. However, the enzyme concentration in this phase system reached only 0.35 U ml<sup>-1</sup> in the top phase. The bacterial cells were microscopically observed to partition totally to the bottom phase in the aqueous two-phase system used. The results are discussed in relation to recirculation of cells by immobilizing to a solid matrix. Extraction of the product to the top phase and the effect of the phase polymers, especially PEG, on the production are also discussed.

**Keywords:** Fermentation;  $\alpha$ -amylase; *Bacillus subtilis*; aqueous two-phase systems

## Introduction

Cell recirculation and continuous operation may result in improved productivities in biotechnical processes involving cells. This can be achieved by, for example, immobilizing cells to solid supports<sup>1</sup> or by using membrane techniques.<sup>2</sup> With the increasing knowledge about immobilization techniques, the production of enzymes and other extracellular products produced by *Bacillus* species with these techniques have been reported:  $\alpha$ -amylase (1,4- $\alpha$ -D-glucan glucanohydrolase, EC 3.2.1.1)<sup>3–4</sup> bacitracin<sup>5</sup> and pro-insulin.<sup>6</sup> However, immobilization to solid supports imposes restrictions in terms of steric hindrances and diffusion limitations, which limits its applicability to low molecular weight substrates.

Aqueous two-phase systems offer another possibility to recirculate living cells. The cells are partitioned to the bottom phase.<sup>7</sup> The cell-free top phase can then be used to accumulate the desired product.

Aqueous two-phase systems are formed when water solutions of two polymers or one polymer and one salt are mixed. They have successfully been used for the large-scale isolation and purification of intracellular enzymes.<sup>8–11</sup> They have also been applied to biological analysis<sup>12</sup> and cofactor regeneration.<sup>13–14</sup> Enzymatic bioconversions in aqueous two-phase systems have been demonstrated for the deacylation of benzylpenicillin<sup>15</sup> and the saccharification of cellulose.<sup>16</sup> Several microbial systems have also been investigated: toxin production with *Clostridium tetani*,<sup>17</sup> conversion of cellulose<sup>18</sup> and starch<sup>7</sup> to ethanol, production of acetone and butanol using *Clostridium acetobutylicum*<sup>19</sup> and cellulase production with *Trichoderma reesei*.<sup>20</sup>

The present investigation concerns the production of a macromolecular product, the enzyme  $\alpha$ -amylase, with *Bacillus subtilis*.

## Materials and methods

### Microorganisms, media and culture conditions

*Bacillus subtilis* was kindly donated by Dr Matthias Uhlen, Royal Institute of Technology, Stockholm, Sweden. The strain is a mutant obtained from *B. subtilis*, Marburg, ATCC 6051.

The organism was grown on a medium containing per litre: 10 g D-glucose, 5 g peptone, 2 g yeast extract (both Difco, Detroit, Michigan, USA), 1.5 g NaCl, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.1 g CaCl<sub>2</sub>. To maintain the strain it was grown for 24 h at 37°C in a reciprocal shaker (175 strokes min<sup>-1</sup>) in a 1 litre baffled Erlenmeyer flask. The medium volume was 100 ml and the pH was adjusted to 7.0 before sterilization. Sterile glycerol was then added to constitute 15% of the volume. This solution was frozen and stored at -70°C.

In the  $\alpha$ -amylase production medium the D-glucose in the medium above was exchanged for soluble starch, 50 g l<sup>-1</sup>. For the cultivations in phase systems the nutrients were calculated as g per kg phase system. This gives a negligible deviation from the calculation on a volume basis.

Production medium (100 ml) was inoculated with the stock culture (5 ml) and cultivated as described above for 16 h. This solution was used as a seed culture for the fermenter cultivations. The fermenter (model FPL-3, Chemofarm AB, Hägersten, Sweden) had a working volume

of 2.5 litre in which 2.5 kg phase system was used. The stirring was set at 300 rev min<sup>-1</sup> and the aeration 1 vvm in all cultivations. pH was maintained at 7.0 during the cultivations with NaOH and HCl, both 3 M. The strength of alkali and acid was chosen to eliminate dilution of the phase system, which might change its volume ratio. A 30% silicone antifoaming agent (BDH Chemicals Ltd, Poole, England) was used dropwise when needed to break the foam.

### Aqueous two-phase system

Dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden) and poly(ethylene glycol) (PEG) of two different molecular weights were used to obtain aqueous two-phase systems. Carbowax PEG 3350 (MW 3000–3700) was obtained from Union Carbide (Danbury, CT, USA) and PEG 600 (MW 570 630) was purchased from Merck (Hohenbrunn, FRG).

The preparation of the aqueous two-phase system consisting of PEG 3350/Dextran T 500 has been described by Albertsson.<sup>21</sup> For the phase system PEG 600, PEG 3350/Dextran T 500 the top-phase composition was determined by measuring the dextran content in a polarimeter (Perkin-Elmer) at 589 nm. Dry weight determination gave the total polymer content. The relation between the content of the two types of PEG was assumed to be the same in the top phase as in the total phase system.

Dextran, which is a carbohydrate, was sterilized separately with the starch to avoid formation of unwanted Maillard products with the nitrogen sources. PEG was sterilized with salts and nitrogen sources. The phase system was mixed directly in the fermenter after the sterilization, because mixing of phase components prior to sterilization results in viscous solutions, which impose heat transfer problems and, furthermore, are difficult to handle. Repeated batch fermentations using aqueous two-phase systems were carried out as follows: when the enzyme production ceased, the aeration and the stirring were turned off and the phases were allowed to separate for 30 min. The top phase was then pumped out through a tube in the top of the fermenter. Fresh top phase and a small volume of total phase system (calculated to compensate for samples taken out during the cultivation) were added aseptically together with nutrients. Fermentation was started again by aerating and stirring the fermenter.

Nutrients were added at the concentrations given above. However, where indicated in the text the volume on which the calculations were based could be either the volume of the added fresh phase system or the total volume in the fermenter.

### Sampling and analysis

Samples taken from the fermenter were centrifuged for 1 min at 5500 rev min<sup>-1</sup> in a bench-scale centrifuge (Wifug Doctor, Stockholm, Sweden) to separate the phases, which were analysed separately.

Cell mass was measured as absorbance at 620 nm in the fermentation broth and in the bottom phase of the phase system, respectively. Viable counts were also determined for the bottom phase. Tryptone Glucose Extract Agar (Oxoid, Basingstoke, England) was used. The incubation was performed for 24 h at 37°C. Dry mass determinations were not performed, because of interference from the added polymers. The cell mass constitutes only a few per cent of the polymer mass.

$\alpha$ -Amylase was analysed with Phadebas (Pharmacia Diagnostics, Uppsala, Sweden).

The starch was analysed with a method in which the starch was first degraded with amyloglucosidase (Sigma Chemical Co., St Louis, MO, USA) for 15 min at 55°C. The D-glucose formed was analysed by the method of Trinder<sup>22</sup> using 4-aminoantipyrine (Sigma Chemical Co., St Louis, MO, USA) as an electron acceptor. Soluble starch was used as a standard.

The partition coefficient for  $\alpha$ -amylase in the aqueous two-phase systems was defined as  $K = \text{activity (top phase)}/\text{activity (bottom phase)}$ . Concentration was used for determination of the partition coefficient for soluble starch.

The total amount of  $\alpha$ -amylase in an aqueous two-phase system was calculated from the partition coefficient and the volume ratio. The total concentration of  $\alpha$ -amylase given in the figures is the ratio between the total amount and the total volume.

## Results

### Partition of *Bacillus subtilis* cells in the aqueous two-phase system

*Bacillus subtilis* cells partition to the bottom phase in an aqueous two-phase system of PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w) (Figure 1). The same partition was observed in the phase system PEG 600, 8% (w/w), PEG 3350, 5% (w/w)/Dextran T 500, 2% (w/w) also used in this study. The emulsion of the bottom phase in the top phase coalesces during the microscopical observation, which makes it hard to determine whether the cells collect at the interface or in the bottom phase. However, Figure 1 clearly shows that the cells are on the bottom-phase side of the interface and that the top phase is free from cells.



Figure 1 *B. subtilis* cells in an aqueous two-phase system composed of PEG 3350, 9% (w/w) and Dextran T 500, 2% (w/w). Cells accumulate in the bottom phase droplets

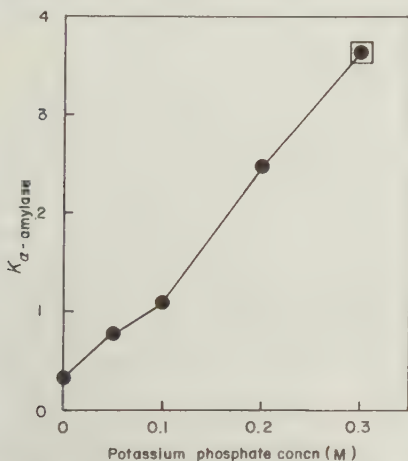


### Partition of $\alpha$ -amylase and starch

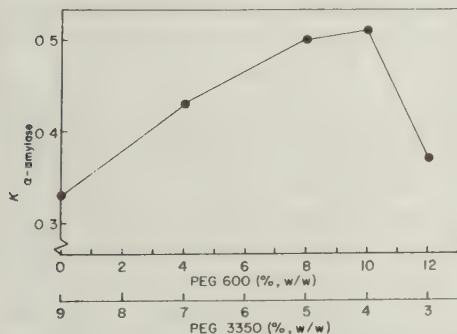
The partition of  $\alpha$ -amylase was studied in the aqueous two-phase system PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w) with increasing potassium phosphate concentrations (Figure 2). An increase of the potassium phosphate concentration from 0 to 0.3 M increased  $K$  from 0.33 to 3.64.

*Bacillus subtilis* does grow in media containing up to 0.2 M potassium phosphate. In an attempt to explore the possibility of using a phase system with 0.2 M potassium phosphate for the production of  $\alpha$ -amylase, this system was stirred (300 rev min<sup>-1</sup>) and aerated (1 vvm) in the fermenter. This resulted in gelling of the dextran<sup>9</sup> and flocculation occurred in the fermenter. Instead, the phosphate concentration in the regular medium, corresponding to 0.04 M, was used in the following experiments.

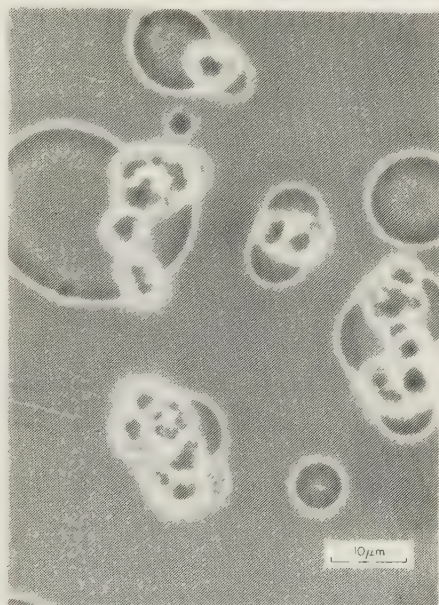
The partition coefficient of  $\alpha$ -amylase could be increased using a mixture of PEG 600 and PEG 3350 as top-phase components (Figure 3). A phase system composed of PEG 600, 8% (w/w), PEG 3350, 5% (w/w) and Dextran T 500, 2% (w/w) gave a partition coefficient for  $\alpha$ -amylase



**Figure 2** Partition coefficient,  $K$ , for  $\alpha$ -amylase in a phase system of PEG 3350, 9% (w/w), Dextran T 500, 2% (w/w), pH 7.5, with various potassium phosphate concentrations.  $\alpha$ , Value from ref. 8



**Figure 3** Partition coefficient,  $K$ , for  $\alpha$ -amylase in a phase system of Dextran T 500, 2% (w/w) and different concentrations of PEG 600 and PEG 3350, pH 7.0



**Figure 4** Precipitated starch particles in the bottom-phase droplets. Aqueous two-phase system as in Figure 1

of 0.5. The volume ratio [volume (top phase)/volume (bottom phase)] of this phase system was 9. Theoretically, 82% of the  $\alpha$ -amylase is partitioned to the top phase in such a system compared with 73% in the phase system PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w).

Soluble starch was used for the enzyme production experiments. The partition coefficient of starch in the phase system PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w) was 0.55 and in the phase system PEG 600, 8% (w/w), PEG 3350, 5% (w/w)/Dextran T 500, 2% (w/w) it was 0.80. These figures refer only to soluble starch, because soluble starch precipitates in the phase system and accumulates in the bottom phase (Figure 4). It is observed as a milky precipitate. However, during the fermentations the  $\alpha$ -amylase produced degrades the starch and the precipitate disappears (Figure 5).

### Production of $\alpha$ -amylase in aqueous two-phase system

*Bacillus subtilis* was first cultivated in a normal growth medium (Figure 6).  $A_{620}$  reached 23 after 112 h. 0.58 U ml<sup>-1</sup>  $\alpha$ -amylase was produced and 38 g l<sup>-1</sup> out of 50 g l<sup>-1</sup> starch was consumed. The overall productivity was  $5.2 \times 10^{-3}$  U ml<sup>-1</sup> h<sup>-1</sup> and during the first 60 h of the cultivation it was  $8.4 \times 10^{-3}$  U ml<sup>-1</sup> h<sup>-1</sup>.

The production of  $\alpha$ -amylase was then studied batch-wise in the aqueous two-phase system PEG 3350, 9% (w/w)/Dextran T 500, 2% (w/w) (Figure 7). This phase system was chosen because the composition of top and bottom phases, respectively, is known.<sup>21</sup> This makes it possible to add fresh top phase with the composition, which is necessary to maintain the phase system when the top phase is changed.

The absorbance reached 58 in the bottom phase after 90 h. The  $\alpha$ -amylase concentration in the top phase was  $0.35 \text{ U ml}^{-1}$ , which corresponds to a total production of  $0.48 \text{ U ml}^{-1}$ . This production is 83% of the production in the normal growth medium (Figure 6). The overall productivity was  $5.3 \times 10^{-3} \text{ U ml}^{-1} \text{ h}^{-1}$ .

In order to improve the production of  $\alpha$ -amylase, a phase system with a higher partition coefficient was studied [PEG 600, 8% (w/w), PEG 3350, 5% (w/w)/Dextran, 2% (w/w)] over two repeated batch experiments. For this phase system the composition of top and bottom phase was not previously known. The composition of the top phase was determined to be PEG 600, 8.4% (w/w), PEG 3350, 5.2% (w/w)/Dextran T 500, 0.8% (w/w) according to the procedure given in Materials and methods.

After removal of the top phase it was assumed that the concentrations of starch and other nutrients remaining in the bottom phase were high enough to support growth and  $\alpha$ -amylase production with *B. subtilis*. Consequently, the concentrations of starch and other nutrients in the freshly added top phase were the same as in the growth medium.

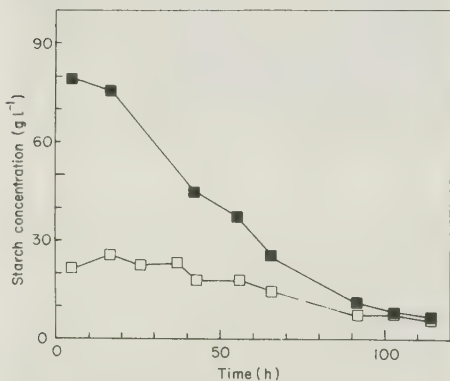


Figure 5 Starch concentration in the top phase (○) and bottom phase (■) of an aqueous two-phase system PEG 600, 8% (w/w), PEG 3350, 5% (w/w), Dextran T 500, 2% (w/w) during a fermentation with *B. subtilis*

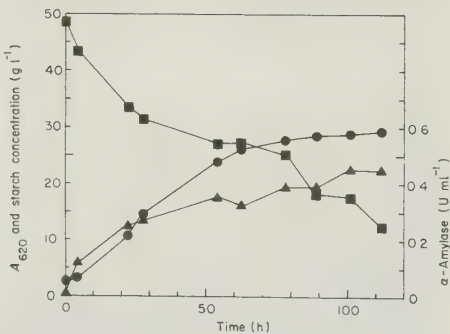


Figure 6 Reference fermentation with *B. subtilis* in normal fermentation medium as described in Materials and methods. ○,  $A_{620}$ ; ●,  $\alpha$ -amylase concentration; ■, starch concentration. Stirring 300 rev min<sup>-1</sup>; aeration 1 vvm; pH 7.0; 37°C

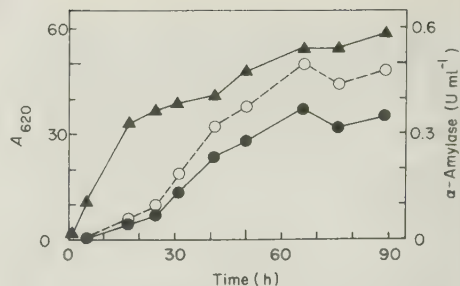


Figure 7 Production of  $\alpha$ -amylase with *B. subtilis* in an aqueous two-phase system [PEG 3350, 9% (w/w), Dextran T 500, 2% (w/w)]. ▲,  $A_{620}$  in the bottom phase; ●,  $\alpha$ -amylase in the top phase; ○, total  $\alpha$ -amylase. All other conditions as in Figure 6

A maximum  $A_{620}$  of 68 and 94, respectively, was reached in the two fermentations (Figure 8). The  $\alpha$ -amylase activity in the top phase was  $0.72$  and  $0.55 \text{ U ml}^{-1}$ , respectively, which corresponds to a total production of  $0.90$  and  $0.46 \text{ U ml}^{-1}$  in the two cultivation steps. The overall productivities were  $7.8 \times 10^{-3}$  and  $5.3 \times 10^{-3} \text{ U ml}^{-1} \text{ h}^{-1}$ . Thus, the production in the second step was only 55% and the productivity only 68% of that obtained in the first step.

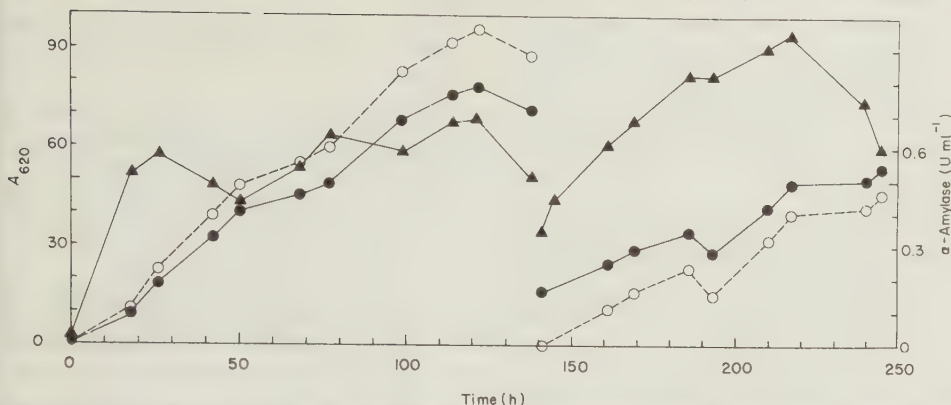
The unfavourable development of enzyme production in the second fermentation step was thought to be due to depletion of one or more essential nutrients. Therefore, nitrogen sources and salts were added with the fresh top phase in proportion to the total fermentation volume, assuming total depletion in the remaining bottom phase. Furthermore, in order to avoid saturation of the bottom phase with precipitated starch, the remaining soluble starch was analysed after each fermentation and starch was added to re-establish an average starch concentration in the whole fermentation volume of  $50 \text{ g l}^{-1}$ . Under these conditions three consecutive batch fermentations were carried out (Figure 9).

In the first two steps an  $\alpha$ -amylase activity of  $0.85 \text{ U ml}^{-1}$  was reached. In the third step the enzyme activity was  $1.35 \text{ U ml}^{-1}$ . The total production of  $\alpha$ -amylase was  $1.03$ ,  $0.80$  and  $1.44 \text{ U ml}^{-1}$ , respectively, in the three steps, which corresponds to overall productivities of  $9.0 \times 10^{-3}$ ,  $7.7 \times 10^{-3}$  and  $16.9 \times 10^{-3} \text{ U ml}^{-1} \text{ h}^{-1}$ . Thus, in each step the  $\alpha$ -amylase production was double that obtained with normal growth medium and double the productivity could be maintained over three consecutive experiments using this aqueous phase system.

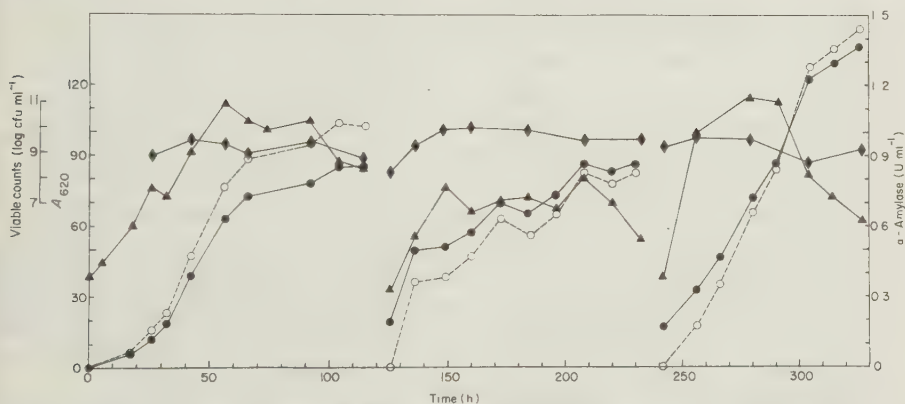
Absorbance measurements might not give correct estimates of cell mass when cells are recirculated, because cells walls from dead cells accumulate in the bottom phase. Viable counts measurements were therefore done in the last three experiments to determine the number of active cells. The viable counts were in the ranges of  $10^{8.9-9.7}$ ,  $10^{8.3-10.2}$  and  $10^{8.7-9.8}$  in the three steps, respectively.

In the normal fermentation (Figure 6) 2400 ml broth can be separated from the cells containing  $0.58 \text{ U ml}^{-1}$  of  $\alpha$ -amylase. This gives a total production of  $1390 \text{ U}$  available enzyme;  $125 \text{ g}$  starch was used for this production. Thus, the yield is  $11.1 \text{ U g}^{-1}$  starch;  $2100 \text{ ml}$  top phase can be separated in each of the three consecutive steps with  $0.85$ ,  $0.85$  and  $1.35 \text{ U ml}^{-1}$  of  $\alpha$ -amylase, respectively;  $354.5 \text{ g}$  of starch was used for this production of a total of  $6405 \text{ U}$   $\alpha$ -amylase. The yield of the available enzyme is  $18.1 \text{ U g}^{-1}$





**Figure 8** Production of  $\alpha$ -amylase with *B. subtilis* in an aqueous two-phase system [PEG 600, 8% (w/w), PEG 3350, 5% (w/w), Dextran T 500, 2% (w/w)]. The composition of the added top phase was PEG 600, 8.4% (w/w), PEG 3350, 5.2% and Dextran T 500, 0.8% (w/w). All other conditions as in Figure 6.  $\blacktriangle$ ,  $A_{620}$  in the bottom phase;  $\bullet$ ,  $\alpha$ -amylase in the top phase;  $\circ$ , total  $\alpha$ -amylase



**Figure 9**  $\alpha$ -Amylase production with *B. subtilis*. The same phase system and the same top-phase composition as in Figure 8. The nutrient concentration in the replaced top phase was calculated on the total volume in the fermenter as described in the text. All other conditions as described in Figure 6.  $\blacktriangle$ ,  $A_{620}$  in the bottom phase;  $\blacklozenge$ , viable counts;  $\bullet$ ,  $\alpha$ -amylase in the top phase;  $\circ$ , total  $\alpha$ -amylase

starch. This is a 63% increase on that of the normal fermentation.

## Discussion

The partition coefficients formed for  $\alpha$ -amylase in the present investigation are in agreement with previously reported data for phase systems containing 0.3 M potassium phosphate.<sup>8</sup> The latter study was performed in order to develop phase systems for the purification of enzymes. However, when a phase system is to be used for microbial production purposes, other considerations have to be taken into account, and the phase system has to be optimized with respect both to the producing organism and the desired product. Therefore, in the present study a phase system with low phosphate concentration, based on a mixture of two types of PEG in the top phase and giving a yield of 82% of total enzyme production, was developed.

The fact that microbial cells partition to the bottom phase in an aqueous two-phase system makes it possible to obtain a cell-free top phase containing the fermentation product. In this respect the aqueous two-phase system can be regarded as an alternative to other methods used to separate cells from a fermentation medium.

The recirculation of living cells in aqueous two-phase system as demonstrated in the present investigation should be compared with other methods of recirculation: flocculation, centrifugation, membrane techniques and immobilization to solid supports. Aqueous two-phase systems have the advantage that they can be designed so that the product-containing top phase is completely free of cells and cell debris. Macromolecular or solid substrates can be used because the phase system does not impose any steric hindrances and diffusion limitations as does, for example, immobilization. Even substrates containing particulate impurities, which might plug a membrane, can be used in an aqueous two-phase system.

Recirculation of cells is often used to achieve an improvement of the productivity of the process. A two-fold increase in productivity was found in the present investigation. Similar results have been reported by Shinmyo *et al.*<sup>4</sup> using *B. amyloliquefaciens* immobilized to a solid support for continuous  $\alpha$ -amylase production. On the contrary, Kokubu *et al.*<sup>3</sup> used immobilized *B. subtilis* in repeated batch fermentations and did not report an increased productivity.

In biotechnical processes, however, productivity might not be the most important factor determining the effectiveness of the process. Of much greater importance might be the final product concentrations. If improved productivity is gained at the expense of a dilute product stream, the cost of concentrating the product may be the limiting factor in the overall economics of the process.

In the present investigation, the  $\alpha$ -amylase concentration in the top phase was almost doubled as compared to the concentration produced in a normal growth medium. In contrast to these results, the improved productivities reported for immobilized *B. amyloliquefaciens*<sup>4</sup> were gained at the expense of an extremely dilute product stream. Only 4% of the enzyme concentration in a normal submerged medium was reached in the effluent from the immobilized system. Kokubu *et al.*<sup>3</sup> reported a concentration only 13% of the concentration in a comparative submerged culture.

The increased enzyme concentration found in the present investigation might be due to the extraction of the product to the top phase. For the two-phase systems studied, as much as 82 and 73% of produced enzyme, respectively, was lost to, i.e. can be recovered from, the top phase. However, the amount of enzyme produced in these two systems differed by more than a factor of two. Therefore, the set-up as an extractive fermentation cannot completely explain the greatly improved enzyme production with *B. subtilis* in certain aqueous two-phase systems.

It has been shown that Tween 80 increases the cellulase production for *Trichoderma* species.<sup>23</sup> Tween 80 has PEG side chains. PEG is also known as a fusion-inducing agent, e.g. it is used for fusion of plant tissue cells.<sup>24</sup>

Thus, the present investigation shows that PEG might improve the release of enzyme from the bacterial cells. However, the finding that improved enzyme production was obtained in only one of the phase systems studied

indicates that the concentration as well as the molecular weight of the polymer influence enzyme production.

## References

- 1 *Methods of Enzymology* (Mosbach, K., ed.), Academic Press, New York, 1976, vol. 46
- 2 Michaels, A. S. *Desalination* 1980, **35**, 329-351
- 3 Kokubu, T., Karube, I. and Suzuki, S. *Eur. J. Appl. Microbiol. Biotechnol.* 1978, **5**, 233-240
- 4 Shinmyo, A., Kimura, H. and Okada, H. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, **14**, 7-12
- 5 Morikawa, Y., Karube, I. and Suzuki, S. *Biotechnol. Bioeng.* 1980, **22**, 1015-1023
- 6 Mosbach, K., Birnbaum, S., Hardy, K., Davies, J. and Bülow, L. *Nature* 1983, **302**, 543-545
- 7 Mattiasson, B. and Hahn-Hägerdal, B. in *Immobilized Cells and Organelles* (Mattiasson, B., ed.), CRC Press, Boca Raton, Florida, 1983, vol. 1, pp. 121-134
- 8 Kroner, K. H., Hustedt, H. and Kula, M.-R. *Biotechnol. Bioeng.* 1982, **24**, 1015-1045
- 9 Kula, M.-R., Kroner, K. H. and Hustedt, H. *Adv. Biochem. Eng.* 1982, **24**, 73-118
- 10 Veide, A., Smeds, A.-L. and Enfors, S.-O. *Biotechnol. Bioeng.* 1983, **25**, 1789-1800
- 11 Veide, A., Lindbäck, T. and Enfors, S.-O. *Enzyme Microb. Technol.* 1984, **6**, 325-330
- 12 Mattiasson, B. and Ling, T. G. I. *J. Immunol. Methods* 1980, **38**, 217-223
- 13 Smeds, A.-L., Veide, A. and Enfors, S.-O. *Enzyme Microb. Technol.* 1983, **5**, 33-36
- 14 Yamazaki, Y., Suzuki, H. *Rep. Ferment. Res. Inst. Jpn* 1979, **52**, 33-40
- 15 Andersson, E., Mattiasson, B. and Hahn-Hägerdal, B. *Enzyme Microb. Technol.* 1984, **6**, 301-306
- 16 Tjerneld, F., Persson, I., Albertsson, P.-Å. and Hahn-Hägerdal, B. *Biotechnol. Bioeng.* in press
- 17 Puziss, M. and Hedén, C.-G. *Biotechnol. Bioeng.* 1965, **7**, 355-366
- 18 Hahn-Hägerdal, B., Mattiasson, B., Andersson, E. and Albertsson, P.-Å. *J. Chem. Technol. Biotechnol.* 1982, **32**, 157-161
- 19 Mattiasson, B., Souminen, M., Andersson, E., Häggström, L., Albertsson, P.-Å. and Hahn-Hägerdal, B. in *Enzyme Engineering* (Chibata, I., Fukui, S. and Wingard, L. B. Jr, eds), Plenum Publishing Corporation, New York, 1982, vol. 6, pp. 153-156
- 20 Persson, I., Tjerneld, F. and Hahn-Hägerdal, B. *Enzyme Microb. Technol.* 1984, **6**, 415-418
- 21 Albertsson, P.-Å. *Partition of Cell Particles and Macromolecules* 2nd ed., Wiley-Interscience, New York, 1971
- 22 Trinder, P. *Ann. Clin. Biochem.* 1969, **6**, 24-27
- 23 Reese, E. T. and Maguire, A. *Dev. Ind. Microbiol.* 1971, **12**, 212
- 24 Benbadis, A. and de Virville, J. D. *Plant. Sci. Lett.* 1982, **26**, 257-264

## $\alpha$ -AMYLASE PRODUCTION WITH BACILLUS AMYLOLIQUEFACIENS IN AQUEOUS TWO-PHASE SYSTEMS AND PEG SOLUTIONS

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### SUMMARY

The  $\alpha$ -amylase production of Bacillus amyloliquefaciens was investigated in aqueous two-phase systems and in PEG 600 solutions. The cells did not partition totally to the bottom phases of the aqueous two-phase systems, and the enzyme production was repressed in both of the aqueous two-phase systems and in PEG 600 solutions. Concomitantly, the cultivation time was prolonged, indicating an increased maintenance function. The surface properties of the cells were investigated by phase partition and compared to the surface properties of Bacillus subtilis, which showed increased  $\alpha$ -amylase production. The cell surfaces of B. amyloliquefaciens became more hydrophobic after growth in a 20%(w/w) PEG 600 solution, whereas the cell surfaces of B. subtilis became more hydrophilic. The results are discussed in relation to the action of PEG on cell walls and cell membranes.

### INTRODUCTION

The production of extracellular enzymes with different strains of the genus Bacillus is an important part of the biotechnical industry. The secretion of the enzymes is very important since it simplifies the downstream processing. The mechanism and regulation of enzyme synthesis and secretion has recently been reviewed by Priest (1).

Several attempts have been made to improve the processes for enzyme production. Continuous production in a two-stage fer-

mentor has been described by Jensen (2). Another approach is to increase the utilization of the cells in order to reduce substrate consumption for cell growth. Immobilization of Bacillus sp. cells to a solid matrix is one example, which has been described both for production of  $\alpha$ -amylase (3-4) and production of proinsulin with B. subtilis containing recombinant DNA (5). This method allows continuous production. The product concentration in the effluent, however, has been reported to be low, which makes the subsequent downstream processing more difficult.

Another method to recycle cells is to use aqueous two-phase systems (6), where the cells are partitioned to the bottom phase and the product can be extracted from a cell free top phase. This has been demonstrated for toxin production by Clostridium tetani (7), production of ethanol from cellulose (8) and starch (9), production of acetone and butanol with Clostridium acetobutylicum (10), cellulase production with Trichoderma reesei (11), and production of  $\alpha$ -amylase with Bacillus subtilis (12).

When  $\alpha$ -amylase ( $\alpha$ -1,4-D-glucan glucanohydrolase, EC 3.2.1.1) was produced in an aqueous two-phase system with B. subtilis the product formation was altered. In one two-phase system the production increased, in another it decreased (12). This indicated that the phase system, as a method for extractive fermentations, was not be responsible for the change in product formation. For Trichoderma sp. the surfactant Tween 80, which has a chemical structure containing poly(ethylene glycol)(PEG), has been found to improve the cellulase production (13). In a recent study on the effects of various surfactants on the  $\alpha$ -amylase production, however, it was found that only PEG 600 improved the enzyme production by B. subtilis (14).

PEG is a wellknown fusogenic agent. It is widely used and described for the fusion of plant protoplasts (15), animal protoplasts (16) and bacterial protoplasts (17-18).

The present investigation has been carried out in order to study whether the  $\alpha$ -amylase production with a technical

strain of Bacillus amyloliquefaciens can be further improved in an aqueous two-phase system and PEG solutions, respectively. The results are discussed in relation to polymer induced alterations of the bacterial cell wall in B. amyloliquefaciens and B. subtilis, respectively.

## MATERIALS AND METHODS

### Organisms and media

Bacillus amyloliquefaciens ALKO 269 was a kind gift from ALKO Ltd, Helsinki, Finland. This strain has been previously described as Bacillus subtilis K160 (19). The production medium contained, in g/L: peptone, 10; yeast extract, 10 (both Difco, Detroit, MI, USA); NaCl, 5;  $\text{KH}_2\text{PO}_4$ , 5;  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 1;  $\text{CaCl}_2$ , 1;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.1; Fe(III)-citrate, 0.1 and soluble starch, 50. To maintain the strain it was grown for 16 hours at 37°C in a reciprocal shaker (175 strokes /min) in a 1 litre baffled Erlenmeyer flask. The medium volume was 100 mL and the pH was adjusted to 7.0 before sterilization. Sterile glycerol was then added to constitute 15 % of the volume. This solution was frozen and stored at -20°C. For the cultivations in aqueous two-phase systems and in PEG solutions, the nutrients were calculated as g per kg medium.

The seed culture for the fermentor cultivations was prepared by adding 5 mL of the stock culture to 100 mL of production medium in a shake flask, followed by 16 hours of cultivation as described above.

Bacillus subtilis was kindly donated by Dr Matthias Uhlen, Royal Institute of Technology, Stockholm, Sweden. This strain has been used in a previous study (12). The medium composition is the same as previously used, in g/L: peptone, 5.0; yeast extract, 2.0; NaCl, 1.5;  $\text{KH}_2\text{PO}_4$ , 0.5;  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.5;  $\text{CaCl}_2$ , 0.1 and soluble starch, 50. The strain was maintained as previously described (12).



### Cultivation conditions

The cultivations were performed in a fermentor (model FPL-3, Chemoferm AB, Hågersten, Sweden) with a 2 litre working volume, or 2 kg when the polymer solutions were cultivated. The stirring was 500 rev/min in all cultivations and the aeration was 1 vvm. The temperature was 37°C and pH was kept between 6.6 and 7.4 with 25 % ammonia and 30 % phosphoric acid. Adekanol LG-109 (Asahi Electro Chemical Co., Japan) was used as antifoaming agent.

### Aqueous two-phase systems and PEG solutions

Dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden) and poly(ethylene glycol)(PEG) of two different molecular weights were used to obtain the aqueous two-phase systems and the different PEG solutions. PEG 600 was purchased from Merck (Hohenbrunn, FRG) and PEG 3350 from Union Carbide (Danbury, CT, USA).

The preparation of the aqueous two-phase systems has been described by Albertsson (6). The determinations of the partition coefficient,  $K$ , for  $\alpha$ -amylase in the phase systems and the volume ratio ( $V_T/V_B$ ) of the phase systems were performed as has been described previously (12). The PEG solutions were mixed on a weight/weight base. The solutions were sterilized separately outside the fermentor and the water loss during the sterilization was compensated for before the medium was mixed in the fermentor.

### Analysis

Samples from the phase systems were separated by centrifugation for 1 min. at 5500 rev/min. in a bench-scale centrifuge (Wifug Doctor, Stockholm, Sweden).

Bacterial growth was measured as the optical density at 620 nm. In the phase systems, the optical density was measured only in the bottom phase.

$\alpha$ -Amylase was analyzed with Phasebas (Pharmacia Diagnostics, Uppsala, Sweden).



#### Determination of surface properties of the cells

The cells were grown in shake flasks as described above. Cells of B. amyloliquefaciens were cultivated for 24 hours in regular growth medium and for 48 hours in 20% (w/w) PEG 600 medium. The B. subtilis cells were cultivated for 72 hours. The insoluble starch particles were allowed to precipitate in the refrigerator overnight. The cells were then centrifuged at 15000 g or more for 20 minutes and washed three times with 0.9% NaCl solution.

Surface properties were investigated by partition in aqueous two-phase systems according to the method described by Gerson and Akit (20). Three phase systems were used, containing PEG 3350 and Dextran T500. The polymer concentrations in the three phase-systems were 6.0/6.0 (%w/w), 7.0/7.0 (%w/w) and 8.0/8.0 (%w/w) for PEG 3350/Dextran T500, respectively. The phase systems also contained 0.01 M NaCl and 0.01 M sodium phosphate, pH 7.0. The partition was performed at 25°C. Cell determination was performed by measuring the optical density after diluting the phase systems and the phases, respectively, with 0.9% NaCl solution.

#### Determination of surface tension and viscosity

The surface tension was determined at room temperature with the "ring method" of de Noüy using a Krüss Tensiometer (Hamburg, FRG).

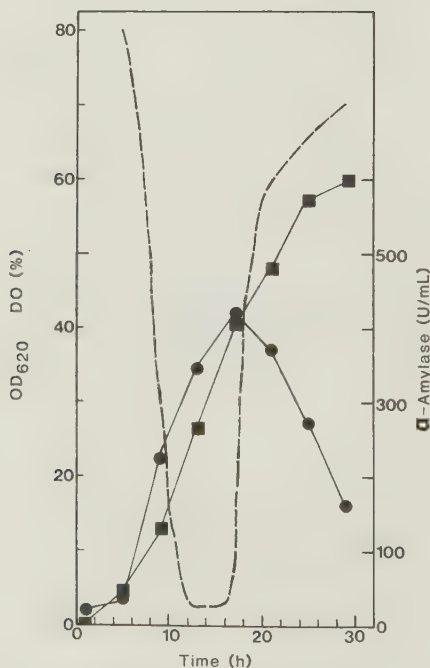
The viscosity measurements were performed in a rotational type viscometer at 25°C. The instrument was a prototype (Bohlin Rheologi AB, Lund, Sweden) coupled to a microcomputer (ABC 806, Luxor, Sweden).

## RESULTS

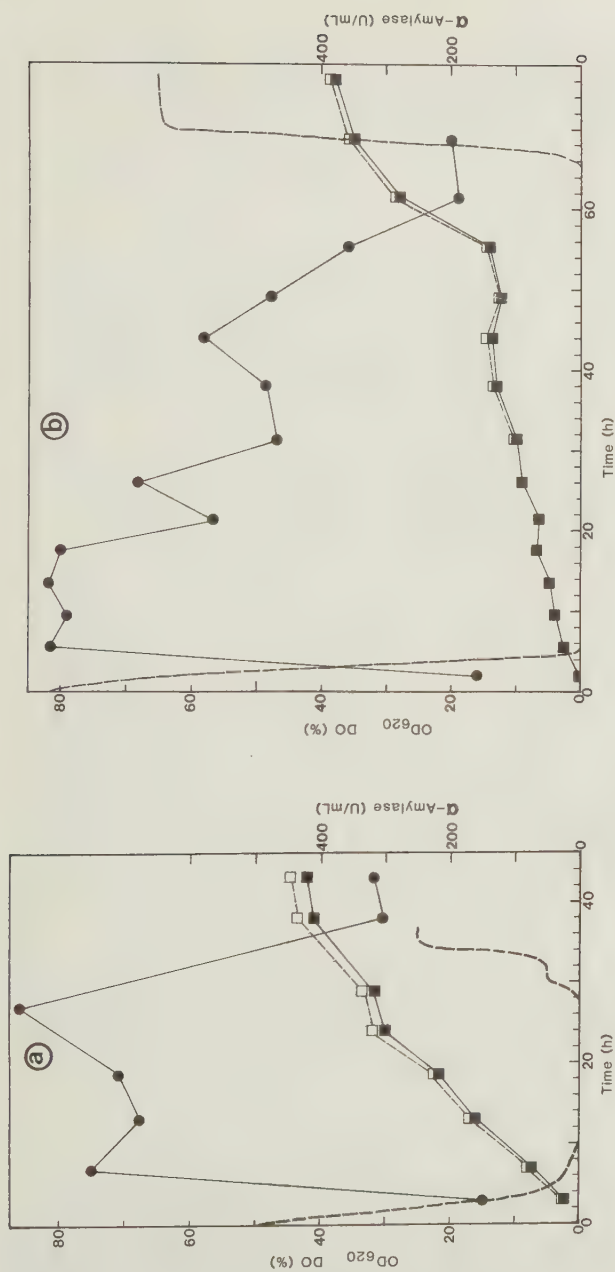
### Production of $\alpha$ -amylase with *Bacillus amyloliquefaciens* in aqueous two-phase systems

*B. amyloliquefaciens* was first cultivated in a regular growth medium (Figure 1). The  $\alpha$ -amylase concentration reached 600 U/ml after 28 hours, corresponding to a productivity of 21 U/ml  $\times$  h. The maximum enzyme concentration was not reached until after a considerable cell lysis had taken place.

The production of  $\alpha$ -amylase was then studied in an aqueous two-phase system composed of 9% (w/w) PEG 3350 and 2% (w/w)



**Figure 1** Reference fermentation of *Bacillus amyloliquefaciens* in medium as described in Materials and methods. (—●—), A<sub>620</sub>; (—■—),  $\alpha$ -amylase concentration; (---), dissolved oxygen. Stirring 500 rev/min; aeration 1 vvm; pH 6.6-7.4; 37°C.



**Figure 2** Production of  $\alpha$ -amylase with *Bacillus amyloliquefaciens* in aqueous two-phase systems: (a) PEG 3350, 9% (w/w)/Dextran T500, 2% (w/w); (b) PEG 600, 8% (w/w)/Dextran T500, 5% (w/w)/Dextran T500, 2% (w/w). (—●—),  $A_{620}$  in the bottom phase; (—■—),  $\alpha$ -amylase in the top phase; (---□---), total  $\alpha$ -amylase. Other symbols and conditions as in Figure 1.

Dextran T500 (Figure 2a), earlier investigated for  $\alpha$ -amylase production with B. subtilis (12). The  $\alpha$ -amylase concentration in the top phase reached 420 U/mL. This corresponds to a total production of 440 U/mL (  $K = 0.62$ ,  $V_T/V_B = 8.8$  ) with a productivity of 10 U/mL x h - half of what was obtained in the reference cultivation.

In order to find out if the production of  $\alpha$ -amylase could be improved, growth and enzyme production was also studied in another phase system, composed of 8% (w/w) PEG 600, 5% (w/w) PEG 3350 and 2% Dextran T500 (Figure 2b). This phase system gave an improved production with B. subtilis (12).

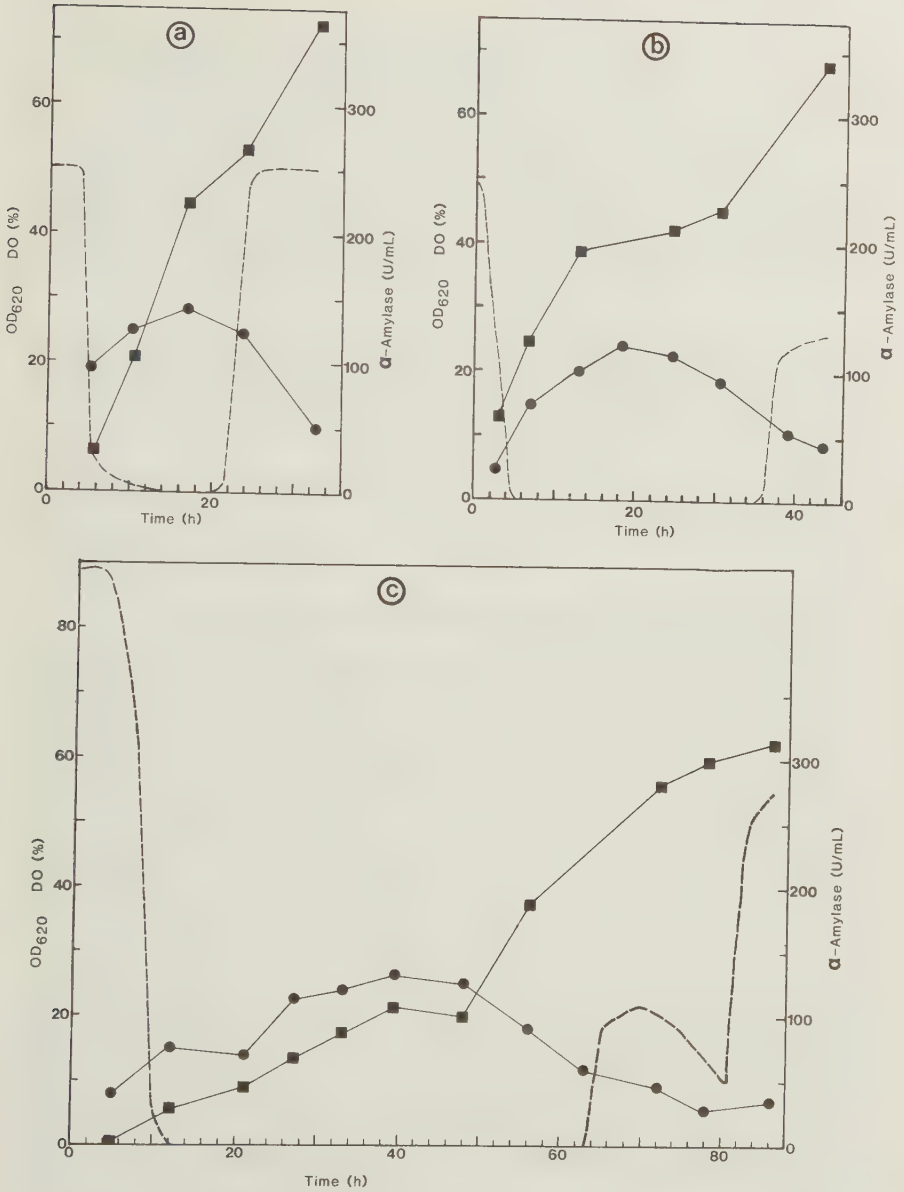
The  $\alpha$ -amylase concentration reached 380 U/mL in the top phase after 76 hours, corresponding to a total production of 390 U/mL ( $K = 0.77$ ,  $V_T/V_B = 9$  ) and a productivity of 5 U/mL x h - one fourth of what was obtained in the reference cultivation.

The level of dissolved oxygen was followed during the fermentation to observe the respiration rate indirectly and to indicate the termination of the fermentations. In the first phase system (Figure 2a), the cells had a high metabolic activity during 30 hours, although the  $\alpha$ -amylase production was repressed. In the reference, the period of high activity lasted only about 15 hours (Figure 1). A marked difference is also observed between the two two-phase systems. In the second system (Figure 2b) the period of high metabolic activity is further extended to more than 60 hours.

An inverse relation seems to exist between enzyme productivity in the three cultivations and the length of the period of high metabolic activity. When the period of high metabolic activity is doubled the enzyme productivity is divided into halves.

#### Production of $\alpha$ -amylase with Bacillus amyloliquefaciens in PEG solutions

In order to further elucidate the influence of PEG on the  $\alpha$ -amylase production with B. amyloliquefaciens, the organism was grown in 10, 15 and 20% (w/w) of PEG 600 (Figure 3a - c).



**Figure 3** Production of  $\alpha$ -amylase with *Bacillus amylolique-faciens* in PEG 600 solutions. (a) 10% (w/w); (b) 15% (w/w); (c) 20% (w/w). Symbols and conditions as in Figure 1.

The presence of PEG inhibited growth so that the optical density only reached levels of 28, 23, and 26, respectively, as compared to 42 in the reference cultivation (Figure 1).

The dissolved oxygen signal indicated that the period of high metabolic activity increased with increasing concentration of PEG; 20, 35 and more than 60 hours, respectively, as compared to 15 hours in the reference cultivation (Figure 1).

The  $\alpha$ -amylase concentration reached 365 U/mL, 340 U/mL and 310 U/mL after 33 hours, 43 hours and 86 hours, respectively. This corresponds to productivities of 11 U/mL x h, 8 U/mL x h and 4 U/mL x h. Again there seems to be an inverse relation between the  $\alpha$ -amylase productivity and the length of the period of high metabolic activity measured as dissolved oxygen.

#### Surface properties of *Bacillus amyloliquefaciens* and *Bacillus subtilis*

Previous investigations on *B. subtilis* cells had shown a one-sided partition of the cells to the bottom phase in both aqueous two-phase systems investigated (12). During the fermentations with *B. amyloliquefaciens* in the two different phase systems, however, a small fraction of the cells partitioned also to the top phase. This has also been observed for other bacilli (21). Cells in phase systems partition according to their surface properties (6), thus suggesting differences in cell surfaces of the two bacilli strains.

Aqueous two-phase systems have been used to separate cells with respect to their surface properties (20). Cells of *B. amyloliquefaciens* and *B. subtilis* were therefore partitioned in three aqueous two-phase systems (Table 1) at 25°C. Preliminary results had shown that all the cells partitioned to the interface when the partitions were performed at +5°C. At higher temperatures a more differentiated partition was obtained.

For both strains the cells from the reference culture are partitioned to the bottom phase and the interphase. Only a



minor fraction is found in the top phase, less so for B. subtilis than for B. amyloliquefaciens.

When the cells are grown in 20% PEG 600, however, there is a striking difference. B. amyloliquefaciens partitions to the top phase, whereas B. subtilis partitions to the bottom phase, indicating that the presence of PEG makes the cell surface of B. amyloliquefaciens more hydrophobic and the cell surface of B. subtilis more hydrophilic.

Microscopic observations of B. amyloliquefaciens grown in 20% PEG 600 frequently revealed elongated cells (Figure 4). Similar observations have been made for B. subtilis (14).

**Table 1** Partition of Bacillus amyloliquefaciens and Bacillus subtilis in aqueous two-phase systems at 25°C as described in Materials and methods.

Polymer concentration:

(PEG 3350 / Dextran T500)(%,w/w) 6/6      7/7      8/8

Bacillus amyloliquefaciens

Reference sample

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 8.1  | 8.3  | 7.7  |
| Bottom phase (%) | 35.5 | 47.5 | 55.8 |

PEG grown cells

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 61.1 | 34.3 | 17.0 |
| Bottom phase (%) | 8.1  | 10.9 | 45.1 |

Bacillus subtilis

Reference sample

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 2.9  | 2.8  | 1.0  |
| Bottom phase (%) | 20.2 | 25.5 | 35.3 |

PEG grown cells

|                  |      |      |      |
|------------------|------|------|------|
| Top phase (%)    | 2.1  | 2.1  | 1.3  |
| Bottom phase (%) | 41.3 | 52.9 | 54.6 |



Figure 4 Bacillus amyloliquefaciens cells from a fermentation in a 20% (w/w) PEG 600 medium. Scale bar, 10  $\mu$ m.

# Surface tension and viscosity

The surface tension and the viscosity were determined in the aqueous two-phase systems and the PEG solutions used in this study (Table 2). The surface tension was decreased with 9.0 to 11.8 mN/m in these solutions compared to pure water. Only the bottom phases of the aqueous two-phase systems had a markedly increased viscosity.

**Table 2** Surface tension and viscosity of aqueous two-phase systems and PEG solutions as described in Materials and methods.

|                                                                       | Surface tension<br>( nN/m ) | Viscosity<br>( mPa·s ) |
|-----------------------------------------------------------------------|-----------------------------|------------------------|
| -----                                                                 |                             |                        |
| <b>Water</b>                                                          | 72.8                        |                        |
| -----                                                                 |                             |                        |
| <b>PEG 600, 8% (w/w) / PEG 3350, 5% (w/w)/ Dextran T500, 2% (w/w)</b> |                             |                        |
| Top phase                                                             | 62.0                        | 4.3                    |
| Bottom phase                                                          | 62.6                        | 82.6                   |
| -----                                                                 |                             |                        |
| <b>PEG 3350, 9% (w/w) / Dextran T500, 2% (w/w)</b>                    |                             |                        |
| Top phase                                                             | 63.0                        | 4.0                    |
| Bottom phase                                                          | 63.8                        | 139.0                  |
| -----                                                                 |                             |                        |
| <b>PEG 600</b>                                                        |                             |                        |
| 10% (w/w)                                                             | 63.5                        | 2.5                    |
| 15% (w/w)                                                             | 62.5                        | 2.9                    |
| 20% (w/w)                                                             | 61.0                        | 3.2                    |
| -----                                                                 |                             |                        |

## DISCUSSION

In two preceeding investigations on  $\alpha$ -amylase production with B. subtilis in aqueous two-phase systems, it was found that the enzyme production was specifically stimulated by PEG 600 (12,14). Neither extraction in phase systems nor surfactants did improve the enzyme production. A better producer of  $\alpha$ -amylase, a strain of B. amyloliquefaciens, was therefore used to investigate if the effect was general among different strains of bacilli. Neither aqueous two-phase systems nor 20% (w/w) PEG 600 improved the enzyme production with this strain. Instead the period of high metabolic activity was prolonged with increasing polymer concentration. A concomitant reduction in enzyme productivity was observed. This indicates a shift from secondary metabolism to maintenance metabolism.

A similar shift in metabolism for Klebsiella pneumonia was previously ascribed to a reduced water activity down to 0.97 in the medium (22). In a screening over different solutes for decreasing water activity down to 0.93, it was found that a decreased water activity inhibited both growth for B. subtilis C-756 and its production of cAMP phosphodiesterase inhibitor (23).

20% (w/w) PEG 600 only decreases the water activity of the growth medium from 0.995 to 0.985 (24), despite the fact that PEG has a high capacity to structure water in the medium, as has been shown with differential scanning calorimetry (25-26).

The oxygen supply is essential for aerobic bacilli. An increased viscosity in a fermentation broth can decrease the oxygen transfer rate (27). Only the bottom phases of the phase systems used in this study have a markedly increased viscosity. The bottom phases, however, only constitute 10 % of the phase systems and are dispersed in the top phases as small droplets ( $\phi < 10 \mu\text{m}$ ). The viscosity in the top phases is much lower.

The oxygen transfer rate,  $OTR = k_L a (c^* - c_L)$ , can be expected to be influenced by the addition of polymers in several ways. The decreased surface tension of the PEG solutions and the phase systems leads to higher values of the interfacial area (against air) per volume,  $a$  (28). The transfer coefficient,  $k_L$ , can be expected to decrease due to the high water binding capacity of PEG (26), thus giving a more rigid interface. The equilibrium dissolved oxygen concentration,  $c^*$ , can also be expected to be altered by the PEG addition, which decreases the polarity of the solution (29). The oxygen solubility is high in e.g. nonpolar substances like paraffines. The total influence on the oxygen transfer rate of these factors is difficult to predict without further investigation.

PEG has been found to partly extract water from membrane bilayers (30). PEG has also been found to make membranes abnormally permeable to ions and molecules (31). Similarly, comparing the effects of fusogenic PEG and electric field treatments on plant protoplasts, the PEG treatment was found to make the protoplasts leaky to intracellular components (32). PEG has also been described to release membrane components rich in lipids from HEP-2 cells (33).

Various physico-chemical properties of PEG solutions have been characterized. The polarity in PEG solutions has been indicated to decrease both from fluorescence measurements (30) and from isotropic hyperfine splitting using ESR spectroscopy (34). A more quantitative parameter for the polar properties of a PEG solution is the dielectric constant, which was reduced when PEG was added to water solutions (29).

The secretion of  $\alpha$ -amylase by Bacillus amyloliquefaciens is dependent on a proton motive force (35). A precursor form of  $\alpha$ -amylase was found to accumulate on the cellular membrane when the electrochemical membrane potential gradient was dissipated by uncouplers. The membrane gradient can thus be expected to be sensitive also to changed dielectric and polar properties of the medium holding increasing concentrations of PEG. It has not been reported whether the dependence for secretion on a proton motive force is similar for both B. amyloliquefaciens and B. subtilis.

In the present study, PEG was found to change the cell wall and/or cell membrane of both B. amyloliquefaciens and B. subtilis. In both cases it led to cell aggregation and cell elongation, similar to the proposed initial step in PEG induced fusion of cells (36). However, it seems the cell surfaces were altered in opposite directions. B. amyloliquefaciens became more hydrophobic and B. subtilis more hydrophilic in response to PEG in the growth medium. Presently the relation between the cell surface hydrophilicity/ hydrophobicity and an increased/ decreased  $\alpha$ -amylase production is not understood.

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#### REFERENCES

1. Priest, F.G. 1984. Aspects of Microbiology 9, Extracellular Enzymes. Coordinating ed., J.A. Cole, C.J. Knowles, and D. Schlessinger. Van Nostrand Reinhold (UK) Co. Ltd, Wokingham.
2. Jensen, D.E. 1972. Continuous production of extracellular protease by Bacillus subtilis in a two-stage fermentor. Biotechnol. Bioeng. 14:647-662.
3. Kokubu, T., I. Karube, and S. Suzuki. 1978.  $\alpha$ -Amylase production by immobilized whole cells of Bacillus subtilis. European J. Appl. Microbiol. Biotechnol. 5:233-240.
4. Shinmyo, A., H. Kimura, and H. Okada. 1982. Physiology of  $\alpha$ -amylase production by immobilized Bacillus amyloliquefaciens. European J. Appl. Microbiol. Biotechnol. 14:7-12.
5. Mosbach, K., S. Birnbaum, K. Hardy, J. Davies, and L. Bülow. 1983. Formation of proinsulin by immobilized Bacillus subtilis. Nature 302:543-545.



6. Albertsson, P.-Å. 1971. Partition of cell particles and macromolecules. 2nd ed., Wiley-Intersciences, New York.
7. Puziss, M., and C.-G. Hede'n. 1965. Toxin production by *Clostridium tetani* in biphasic liquid cultures. Biotechnol. Bioeng. 7:355-366.
8. Hahn-Hägerdal, B., B. Mattiasson, E. Andersson, and P.-Å. Albertsson. 1982. Soluble temporarily immobilized biocatalysts. J. Chem. Tech. Biotechnol. 32:157-161.
9. Mattiasson, B., and B. Hahn-Hägerdal. 1983. Utilization of aqueous two-phase systems for generating soluble immobilized preparations of biocatalysts, p. 121-134. In B. Mattiasson (ed.), Immobilized cells and organelles, Vol. 1. CRC Press, Boca Raton, Florida.
10. Mattiasson, B., M. Souminen, E. Andersson, L. Häggström, P.-Å. Albertsson, and B. Hahn-Hägerdal. 1982. Solvent production by *Clostridium acetobutylicum* in aqueous two-phase systems, p. 153-155. In I. Chibata, S. Fukui, and L.B. Wingard, Jr.(ed.), Enzyme Engineering, Vol. 6. Plenum Publishing Corporation.
11. Persson I., F. Tjerneld, and B. Hahn-Hägerdal. 1984. Semicontinuous cellulase production in an aqueous two-phase system with *Trichoderma reesei* Rutger C 30. Enzyme Microb. Technol. 6:415-418.
12. Andersson, E., A.-C. Johansson, and B. Hahn-Hägerdal. 1985.  $\alpha$ -Amylase production in aqueous two-phase systems with *Bacillus subtilis*. Enzyme Microb. Technol. 7:333-338.
13. Reese, E.T., and A. Maguire. 1971. Increase in cellulase yields by addition of surfactants to cellobiose cultures of *Trichoderma viride*. Dev. Ind. Microbiol. 12:212-224
14. Ramgren, M., E. Andersson, and B. Hahn-Hägerdal. The effect of poly(ethylene glycol) and surfactants on the production of  $\alpha$ -amylase with *Bacillus subtilis*.(manuscript in preparation)
15. Wallin, A., K. Glimelius, and T. Eriksson. 1974. The induction of aggregation and fusion of *Daucus carota* protoplasts by polyethylene glycol. Pfl. Physiol. 74:64-80.
16. Pontocorvo, G. 1975. Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somat. Cell Genet. 1:397-400.
17. Foder, K., and L. Alföldi. 1976. Fusion of protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. USA 73:2147-2150.
18. Schaeffer, P., B. Cami, and R.D. Hotchkiss. 1974. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA 73:2151-2155.

19. Markkanen, P.H., and M.J. Bailey. 1974. Simultaneous production of  $\alpha$ -amylase,  $\beta$ -glucanase and proteolytic enzymes by *Bacillus subtilis*. J. appl. Chem. Biotechnol. 24:93-103.
20. Gerson, D.F., and J. Akit. 1980. Cell surface energy, contact angles and phase partition. II. Bacterial cells in biphasic aqueous mixtures. Biochim. Biophys. Acta 602:281-284.
21. Kantelinen, A., K. Poutanen, M. Linko, and P. Markkanen. 1986. Semicontinuous enzyme production in aqueous two-phase system. (submitted for publication).
22. Esener, A.A., G. Bol, N.W.F. Kossen, and J.A. Roels. 1981. Effect of water activity on microbial growth, p. 339-344. In M. Moo-Young, C.W. Robinson and C. Vezina (ed.), Advances in biotechnology, Vol. 1. Pergamon Press, Toronto.
23. Hosono, K., and B. Hahn-Hägerdal. 1986. Effects of water activity on the growth and the production of cAMP phosphodiesterase inhibitor with *Bacillus subtilis*. J. Appl. Microbiol. Biotechnol. (in press).
24. Andersson, E., and B. Hahn-Hägerdal. 1986. Enzyme action in polymer and salt solutions: 1. Stability of penicillin acylase in PEG and potassium phosphate solutions in relation to water activity. (submitted for publication)
25. Blow, A.M.J., G.M. Botham, D. Fisher, A.H. Goodall, C.P.S. Tilcock, and J.A. Lucy. 1978. Water and calcium ions in cell fusion induced by poly(ethylene glycol). FEBS Letters 94:305-310
26. Tilcock, C.P.S., and D. Fisher. 1982. The interaction of phospholipid membranes with poly(ethylene glycol) vesicle aggregation and lipid exchange. Biochim. Biophys. Acta 688:645-652.
27. Wang, D.I.C., C.L. Cooney, A.L. Demain, P. Dunnill, A.E. Humphrey, and M.D. Lilly. 1979. Fermentation and enzyme technology. John Wiley & Sons, New York.
28. Bailey, J.E., and D.F. Ollis. 1977. Biochemical engineering fundamentals. McGraw-Hill Kogakusha, Ltd, Tokyo.
29. Arnold, K., A. Herrmann, L. Pratsch, and K. Gawrisch. 1985. The dielectric properties of aqueous solutions of poly(ethylene glycol) and their influence on membrane structure. Biochim. Biophys. Acta 815:515-518.
30. Arnold, K., L. Pratsch, and K. Gawrisch. 1983. Effect of poly(ethylene glycol) on phospholipid hydration and polarity of the external phase. Biochim. Biophys. Acta 728:121-128.

31. Aldwinckle, T.J., Q.F. Ahkong, A.D. Bangham, D. Fisher, and J.A. Lucy. 1982. Effects of poly(ethylene glycol) on liposomes and erythrocytes permeability changes and membrane fusion. *Biochim. Biophys. Acta* 689:548-560.
32. Hahn-Hägerdal, B., K. Hosono, A. Zachrisson, and C.H. Bornman. 1986. Polyethylene glycol and electric field treatment of plant protoplasts: characterization of some membrane properties. (submitted for publication).
33. McCammon, J.R., and V.S.C. Fan. 1979. Release of membrane constituents following polyethylene glycol treatment of HEp-2 cells. *Biochim. Biophys. Acta* 551:67-73.
34. Herrmann, A., L. Pratsch, K. Arnold, and G. Lassmann. 1983. Effect of poly(ethylene glycol) on the polarity of aqueous solutions and on the structure of vesicle membranes. *Biochim. Biophys. Acta* 733:87-94.
35. Mure'n, E.M., and L.L. Randall. 1985. Export of  $\alpha$ -amylase by *Bacillus amyloliquefaciens* requires proton motive force. *J. Bacteriol.* 164:712-716.
36. Fisher, D., and A.H. Goodall. Membrane fusion by viruses and chemical agents. *Techniques Cell. Physiol.* P115:1-36.





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